Universidade de Aveiro Departamento de Biologia
2012

Jacinta Maria Marques de Oliveira

Salubridade microbiológica dos bivalves - relevância na segurança alimentar

Microbiological quality of bivalves - relevance to food safety

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor Mário Pereira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro, e co-orientação da Professora Doutora Maria Ângela Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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Recomeça
Se puderes
Sem angústia
E sem pressa.
E os passos que deres,
Nesse caminho duro
Do futuro
Dá-os em liberdade.
Enquanto não alcances
Não descanses.
De nenhum fruto queiras só metade.
E, nunca saciado,
Vai colhendo ilusões sucessivas no pomar.
Sempre a sonhar e vendo
O logro da aventura.
És homem, não te esqueças!
Só é tua a loucura
Onde, com lucidez, te reconheças…

Miguel Torga

Aos meus pais e ao meu marido.

To my parents and to my husband.

o júri / the jury

presidente / president

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palavras-chave Salubridade microbiológica; Bivalves salubres; Segurança alimentar.

resumo

Os moluscos bivalves constituem um recurso haliêutico de elevada importância na economia (inter)nacional pelas suas características organolépticas, valor nutritivo e relevância na gastronomia tradicional. Não obstante, representam um produto alimentar de elevado risco para a saúde pública. A contaminação microbiológica (autóctone e antropogénica), sendo crónica nos bancos de bivalves das zonas estuarino-lagunares, constitui uma das principais preocupações associadas à segurança alimentar. Aquando da filtração inerente aos processos de respiração e alimentação, os bivalves bioacumulam passivamente microrganismos incluindo os patogénicos. A sua colocação no mercado impõe pois, prévia salubrização para níveis microbiológicos compatíveis com a legislação em vigor, salvaguardando a saúde pública. Apesar da monitorização das áreas de apanha e produção, das medidas de prevenção e da depuração, a ocorrência de surtos associados ao consumo de bivalves tem aumentado. Tal deve-se à insuficiente monitorização da contaminação microbiológica dos bivalves, contribuindo para uma gestão ineficaz do produto e consequente sub-valorização.

O presente trabalho pretendeu caracterizar o estado de desenvolvimento do sector de exploração de bivalves em Portugal do ponto de vista da segurança alimentar, e analisar os aspectos cruciais da monitorização e da depuração do produto apresentando alternativas abrangentes e aplicáveis ao sector. Assim, desenvolveu-se uma metodologia de base molecular passível de adaptação à monitorização dos bivalves das zonas conquícolas, como alternativa ao método de referência vigente do Número Mais Provável que é baseado apenas na quantificação de *Escherichia coli*. O mexilhão (*Mytilus edulis*) da Ria de Aveiro, bivalve de interesse comercial a nível (inter)nacional serviu de modelo para a comparação de protocolos de extração de DNA. Esta metodologia foi desenvolvida de modo a que os métodos de extração de DNA sejam passíveis de aplicação a outras matrizes biológicas ou ambientais. Para além da detecção e quantificação directa de bactérias patogénicas, esta metodologia poderá ser aplicada à monitorização da transferência vertical microbiana nos bancos de bivalves bem como à caracterização da dinâmica espacio-temporal das populações microbianas no ambiente e à monitorização dos processos de depuração. Foi ainda abordado o potencial da aplicação de bacteriófagos ou de enzimas líticas para a optimização dos processos de purificação.

O trabalho realizado e as perspectivas futuras propostas pretendem contribuir para a dinamização e requalificação do sector de exploração de bivalves através da melhoria do nível de segurança alimentar dos moluscos bivalves comercializados para alimentação humana, valorizando este recurso.

keywords Microbiological quality; Safe bivalves; Food safety.

abstract

Bivalve molluscs stand out among fishery products, because of their (inter)national economic importance. For organoleptic and nutritional characteristics, because of culture, tradition and food availability reasons, bivalves are highly appreciated by consumers. Notwithstanding they represent a high risk food product. Microbial contamination (autochthonous and anthropogenic) is a major concern associated with the food safety of shellfish since it is known to be chronic in the shellfish beds of estuarine areas. By filter-feeding, bivalves passively accumulate microorganisms including pathogens. Products safe for sale and consumption must accomplish legislation standards preventing infections in the consumers and safeguarding public health. Despite monitoring of shellfish harvesting and production areas, the imposed preventative measures and the depuration processes, the occurrence of outbreaks associated with consumption of shellfish has increased. This may be due to underestimated assessment of microbial contamination of shellfish leading to low quality products.

This work aimed to characterize the state of development of the sector of exploitation of bivalves in Portugal and to analyze the some aspects of bivalve monitoring and purification that lead to underestimation of microbiological contamination, in the perspective of providing comprehensive alternatives applicable to the sector.

A molecular based approach that can be used in the monitoring of shellfish was developed as an alternative to the current reference method, the Most Probable Number based on the quantification of *Escherichia coli* only. Mussels (*Mytilus edulis*) from the *Ria de Aveiro* were used as model bivalves because they are widely represented in the national and international markets. This methodology used an innovative processing of bivalves along with extraction and detection methods that can be used in other biological and environmental matrices. The proposed methodology can be applied not only to direct detection and quantification of pathogenic bacteria, but also to the analysis of vertical tranference of microorganisms in growing beds and to the assessment of spatial and temporal dynamics of microbial populations. Also, it can be used to the classification of harvesting and production areas and to the evaluation of current purification processes. Also, it was suggested the use of bacteriophages and litic enzymes as an adjuvant for the improvement of the actual depuration processes.

The revision of data on this field of activity and the experimental results were integrated in the perspective of providing scientific basis for the sustainable development of bivalve exploitation and valorization of the products in terms of food safety and economic profitability.

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Published papers

Oliveira, J., Cunha, A., Castilho, F., Romalde, J.L. & Pereira, M.J. (2011). Microbial contamination and purification of bivalve shellfish: Crucial aspects in monitoring and future perspectives - A mini-review. *Food Control* 22(6): 805-816.

Oliveira, J., Castilho, F., Cunha, A. & Pereira, M.J. (2012). Bacteriophage therapy as a bacterial control strategy in aquaculture. *Aquaculture International*. doi: 10.1007/s10499-012-9515-7.

Submitted papers

Oliveira, J., Castilho, F., Cunha, A., Pereira, M.J. (submitted). Bivalve harvesting and production in Portugal – an Overview. *Reviews in Fisheries Science.*

Oliveira, J., Cunha, A., Almeida, A., Castilho, F., Pereira, M.J. (submitted). Modified methodology for the extraction of bacterial DNA from mussels – relevance for food safety. *Food Analytical Methods.*

General introduction

Scope of the thesis

1. THE NEED FOR GREATER MICROBIAL CONTROL OF BIVALVES

Seafood is an essential part of the diet of many populations around the world. In some countries, tradition is the main reason for the consumption of shellfish. In the present, the nutritional and health benefits of eating shellfish are also being recognized (Murchie *et al.*, 2005). Shellfish are as nutritious as finfish, milk or eggs and are low in cholesterol and a valuable source of omega-3 fatty acids (Bernardino, 2000; Fauconneau, 2002; Murchie *et al.*, 2005; FAO, 2006; Sapkota *et al.*, 2008).

The global tendency in the growth of human consumption of seafood will undoubtedly continue and the need for increased production will persist as the human populations expand. In recent years, the culture of bivalve molluscs has became an important and rapidly expanding area of aquaculture sector worldwide (Table 1). Some bivalves are wild captured, while the majority of bivalves are originated from aquaculture using seeds either produced from natural populations collected at natural breeding areas or from hatcheries of propagation production (Anrooy *et al.*, 2006).

Table 1 Value for the global production of bivalve by sector and by species, from 1995 to 2005 (in 1000 tonnes) (WHO, 2010).

Year	Sector		Total	Species		
	Aquaculture	Wild		Oyster	Clams, cockles, ark shells	Scallops, pectens
1995	7077,1	1936,6	**9013,7**	3243,1	2726,8	1690,8
1996	7188,7	1845,6	**9034,3**	3223,8	2700,4	1811,3
1997	7406,0	1771,4	**9177,4**	3664,8	2755,5	1802,0
1998	8013,7	1790,7	**9804,4**	3699,9	3100,0	1429,0
1999	8878,8	1831,1	**10709,9**	3878,6	3601,9	1564,2
2000	9156,2	1985,0	**11141,2**	4247,0	3431,8	1815,2
2001	9920,0	2000,8	**11920,8**	4403,8	3933,8	1921,9
2002	10419,5	2018,0	**12437,5**	4504,1	4256,5	1968,1
2003	11217,1	2086,2	**13303,3**	4669,2	4712,4	2023,0
2004	11650,4	1964,5	**13614,9**	4757,4	4944,9	1953,8
2005	11861,9	1726,3	**13588,2**	4781,4	4881,6	1986,2

Bivalves are ideal animals for aquaculture: they are filter feeders and therefore do not require additional feeding, apart from the nutritional resources in the surrounding water, and generally minimum husbandry (Berthe, 2005; Anrooy *et al.*, 2006; FAO, 2006). Although they have been cultured for hundreds of years, recent advances in culture technology have led to a significantly enhancement of the production. Continued improvements in culture methodology and technology are required to meet the increasing demands of the consumers and also to make the culture of bivalves more attractive from the economic perspective to both producers and investors. The main concerns about the bivalve industry are related to the pre-harvest stages (WHO, 2010). Production processes in aquaculture worldwide (e.g. submergible cages, sea ranching, intensification, aquaponics and recirculation systems) sometimes increase vulnerability of bivalves to microbial disease outbreaks and overcoming this generally requires large investments (Anrooy *et al.*, 2006). Also, management of harvesting areas where shellfish farming can be undertaken by monitoring microbial contamination (bacteria, viruses), blooms of biotoxin producing microalgae and pollution remains problematic. In all cases, bivalves become a non-suitable product for human consumption.

Greater efficiency in the processes associated to the management and monitoring of growing areas will become even more valuable in the future, as natural areas where shellfish farming can be undertaken are limited in the world, sometimes with uncertain reliability, and may become even more restricted due to the conflict over the use of the coastal zone as human populations and coastal urbanization grow (Helm & Bourne, 2004; Anrooy *et al.*, 2006). This is a reality of many producing countries which are failing to meet the strict requirements imposed by consuming nations (WHO, 2010).

1.1. MICROBIAL CONTAMINATION AND PUBLIC HEALTH AWARENESS

The hazard posed by shellfish is related to the traditional consumption of bivalves in raw or only mildly cooked dishes (Romalde *et al.*, 1994; Lees, 2000; Murchie *et al.*, 2005). Bivalves bioaccumulate harmful microorganisms namely viruses, bacteria, parasites and microalgae (biotoxins producers), serving as vehicles of these microorganisms and mostly causing gastroenteritis as consequence of their consumption (Defossez & Hawkins, 1997;

Burkhardt & Calci, 2000; Huss *et al.*, 2000; Lees, 2000; Hallegraeff *et al.*, 2003; Dunphy *et al.*, 2006; Botana, 2008).

The earliest reports of shellfish-transmitted bacterial diseases were documented in the late 19th and early 20th century (Potasman *et al.*, 2002). The majority of reported seafood associated illnesses has undefined etiologies and is often assumed to be viral. While viruses are frequently the cause of seafood-related infections, hospitalisations and deaths are especially and generally related with bacteria (Potasman *et al.*, 2002; Butt *et al.*, 2004). Most of the infectious outbreaks cause acute symptoms and a few, generally associated with Noroviruses, Hepatitis A viruses and *V. vulnificus* infections, can be fatal (Potasman *et al.*, 2002). *Vibrio* species (such as *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Vibrio vulnificus*, *Vibrio mimicus*, *Vibrio hollisae*) account for 20% of all outbreaks of disease (Hood & Ness, 1982; Ripabelli *et al.*, 1999; Butt *et al.*, 2004; Normanno *et al.*, 2006). Other bacteria pointed as the etiological agent of shellfish poisoning are *Plesiomonas shigelloides*, *Listeria monocytogenes*, *Escherichia coli* and *Campylobacter*, *Salmonella* and *Shigella* species (Butt *et al.*, 2004; Brands *et al.*, 2005). Although these bacteria are commonly implicated in seafood diseases, they are only occasionally traced to seafood (Potasman *et al.*, 2002). First, some of the bacteria are autochthonous existing in the fresh water and estuaries. Naturally occurring bacteria are the most often cited causative agents of disease and death related to shellfish consumption (Wittman & Flick, 1995; Huss *et al.*, 2000; Croci *et al.*, 2002). Indeed, contamination by autochthonous bacteria represents an additional challenge in achieving edible bivalves (Jones *et al.*, 1991; Croci *et al.*, 2002). Second, the most frequent clinical syndrome (self-limiting gastrointestinal symptoms as gastroenteritis) can be related to a wide range of etiological agents and their laboratory identification requires several laborious tests (Molnar *et al.*, 2006). The increased consumption of bivalves worldwide has been accompanied by numerous reports of infectious seafood outbreaks from almost all continents. The most commonly implicated bivalves are oysters, followed by clams and mussels, since they are also the most consumed bivalves (Potasman *et al.*, 2002).

The government provides legislation and preventive actions in order to ensure consumer protection against seafood poisoning. Despite this continuous effort, the consumers, producers and dealers must be well informed about the hazards of seafood consumption. In many countries, efforts have been initiated to educate fishermen, consumers, distributors,

and health services on the hazard associated to the ingestion of raw shellfish (Potasman *et al.*, 2002). The risk of disease or death due to contaminated shellfish consumption is inherent to all consumers but it increases in those that suffer from underlying health disorders and are exposed to the consumption of raw bivalves. Among the high-risk population are individuals with immunosuppressive disorders (cancer, AIDS), achlorhydria and epilepsy, diabetes mellitus, liver and chronic kidney disease and asthma. Pregnancy, age and alcohol abuse are also factors that may enhance the development of seafood diseases (Ripabelli *et al.*, 1999; Butt *et al.*, 2004). Physicians who care for patients with these underlying health disorders and reduced immunity should be able to provide information on the hazard associated with the ingestion of raw shellfish and advise patients against this type of diet. Fishermen and other agents related to the handling of bivalves are also an important focal point of information dissemination since non-ethical activities such as illegal harvesting from polluted and restricted areas, wet storage of harvested shellfish in polluted waters, and other violations of legislation become problematic when authorized shell fishing harvesting areas decrease (Jones *et al.*, 1991).

2. MONITORING THE GROWING AREAS: OPPORTUNITIES AND CHALLENGES

Human health problems associated with bivalve shellfish are well recognized internationally. The fact that outbreaks of infection continue to occur despite apparently adequate control measures highlights the role of microbial contamination of bivalves (Potasman *et al.*, 2002). Numerous factors contribute to the existence of shellfish-associated diseases outbreaks. Problems regarding monitoring shellfish growing areas and post-harvest contamination of the product (during handling, storing, processing, labeling, and shipping), as well as the lack of consumer education and public awareness are on the basis of diseases outbreaks (Potasman *et al.*, 2002; WHO, 2010). The control of the risk of disease requires an integrated management and monitoring of the shellfish growing areas together with post-harvest product purification and processing (WHO, 2010).

Monitoring of shellfish beds, ultimately intends to verify if the area complies with all the requirements before collection of bivalves for human consumption and to delineate the possible need of further processing prior to sale (Lees, 2000).

The presence of contamination is detected in samples, namely the end-product (shellfish flesh and intravalvular liquid) or environmental water, collected at the harvesting or production sites (Lees, 2000). Usual sources of microbial pollution include sewage effluents without the appropriated treatment, small river outlets or diffuse land runoff, septic tank leakages and other sources of human and animal fecal pollution (Hernroth *et al.*, 2002). Remediation requires the source of contamination to be identified, quantified and measurements to reduce the impact of the contamination to be addressed (Shumway & Rodrick, 2009). Therefore, periodic surveys are needed to assess the sanitary status of the area and to evaluate the impacts of possible changes or the impacts of potential developments of the surrounding area. Along with sanitary surveys, the identification of pollutant sources and estimates on their quantity provide the additional information needed for leading the decision on when to restrain or release sites of harvesting or production areas (IPIMAR, 2008; Shumway & Rodrick, 2009). In the design and implementation of the microbiological monitoring programme the following aspects must be well specified:

1. type of sample (matrix: seawater or shellfish; shellfish species);
2. location for collecting the sample (with latitude allowed around the defined point);
3. number of samples to be taken per sample type per year;
4. periodicity of sampling;
5. depth, tidal state, other environmental factors;
6. method of sample analyses;
7. action to be taken (if any) in the event of samples exceeding the criteria for the class of area.

The results obtained will depend on these defined criteria and will determine at first, a provisional classification and lastly, a full classification (Anonymous, 2004b; WHO, 2010). The European standards for bivalve shellfish beds are summarized in Table 2. Legislation determines that these European standards are based on the microbial analysis of shellfish Flesh and Intravalvular Liquid (FIL) through the detection of bacterial indicators of sanitary quality (*Escherichia coli*) quantified by a 5-tube 3-dilution Most Probable Number (MPN) test. The value of indicator microorganisms to predict the risk associated to other important microbial contaminants in bivalves, was questioned and it is now well established that correlation between the presence of indicator bacteria and viruses or autochthonous bacteria does not always occur (Son & Fleet, 1980; Hood & Ness, 1982;

Romalde *et al.*, 2002; Formiga-Cruz *et al.*, 2003; Marino *et al.*, 2005; Murchie *et al.*, 2005). Also, the conventional methodology used has proven to have critical failures in assessing the sanitary quality of shellfish (Hackney *et al.*, 1979; Rompré *et al.*, 2002). Surveys on the sanitary quality of shellfish flesh or water are being done independently without establishing possible links (Lees, 2000). Therefore, the basic science underlying the monitoring processes must be reappraised and evaluated, particularly exploring the relations between the surrounding environment and bivalve quality. Once such links have been identified, it may be possible to establish relations between the contamination in bivalve flesh and in the environmental samples allowing for a better prediction of a possible contamination and enabling the anticipation of measures for remediation of contaminations. In addition, efforts to understand differences among species and to establish which environmental factors mostly contribute to pathogen biaccumulation will also lead to a better evaluation and more efficient approaches for prevention and mitigation of shellfish contamination (Hernández-Zárate & Olmos-Soto, 2006; La Valley *et al.*, 2009).

Table 2 European classification of bivalve growing areas according to the Most Probable Number (MPN) of *Escherichia coli* per 100 g of bivalve Flesh and Intravalvular Liquid (FIL) (Donovan *et al.*, 1988; Anonymous, 2004b, a; ISO, 2004; Anonymous, 2005, 2008).

Category	MPN of *Escherichia coli* per 100g of bivalve FIL	Treatment required
A	≤230	Direct human consumption.
B	]230; 4 600]	Depuration or relaying, to meet category A.
C	]4 600; 46 000]	Protracted relaying to meet category A with further depuration or cooking by an approved method.

There is widespread agreement on the need to classify growing sites, but divergence occurs when considering the most appropriate methods and the samples to be used (Lees, 2000; IPIMAR, 2008; WHO, 2010). Microbial analysis of the water column may be potentially easier and cheaper, but it relies on the understanding the horizontal transference of

contaminants from water to shellfish flesh. The hypotheses of considering shellfish flesh for the analysis of the microbial contamination is more labourious, expensive and requires larger amounts of product, but provides a more direct assessment of the risk and a better measure of sanitary quality, so it is presently the most used matrix and the type of sample most approved by regulators (IPIMAR, 2008; Shumway & Rodrick, 2009; WHO, 2010).
Progresses in these issues should lead to a better monitoring of shellfish beds, probably with the development of risk management strategies based on more accurate standards.

3. MOLECULAR APPROACHES AS IMPORTANT TOOLS IN MONITORING CONTAMINATION OF BIVALVES

The accurate knowledge of the composition of the bacterial community present in bivalves and their surrounding environment, as well as the complex interactions between them are crucial to control microbial safety of bivalves (Hernández-Zárate & Olmos-Soto, 2006). This will provide insights on sources of water pollution and quality of estuarine environment, and may assist with the development of new purification strategies directed to shellfish-borne human pathogens (La Valley *et al.*, 2009). This contextualization will enable to prevent economic losses that seriously affect of the sector of bivalve production (Hernández-Zárate & Olmos-Soto, 2006).
Current legislation determines that the microbiological health of the bivalves should be determined by culture dependent methods (Lees, 2000; Gugliandolo *et al.*, 2010; Oliveira *et al.*, 2011). Within Europe, the technique most commonly used for the enumeration of *E. coli* in shellfish is Most Probable Number (MPN). The reference method for testing *E. coli* in shellfish is ISO TS 16649-3 based on the method developed by Donovan and collaborators (Donovan *et al.*, 1988; ISO, 2004). This method comprehends two stages. The first stage of the method is an enrichment requiring inoculation of Minerals Modified Glutamate Broth (MMGB) with shellfish homogenates in a series dilution (three dilutions) of five tubes, and incubation at 37±1ºC for 24±2 hours. The presence of *E. coli* is subsequently confirmed by subculturing acid producing tubes onto agar containing 5-bromo-4-chloro-3-indoly-ß-D-glucuronide (X-glucuronide) and detecting growth of blue/green colonies of bacteria on this chromogenic Tryptone Bile X-glucuronide Agar (TBX).

Notwithstanding the helpful value of these procedures, they are selective for the search of bacterial indicators or a specific pathogen (Gugliandolo *et al.*, 2010). Molecular technologies have shown to be appropriate for environmental samples and food products (Thompson *et al.*, 2005) and to complement or replace culture-based approaches and bypass some of its intrinsic biases and limitations (Coyne *et al.*, 2004; Gugliandolo *et al.*, 2010; Oliveira *et al.*, 2011). Molecular techniques may be more sensitive, specific and allow the rapid detection, identification and quantization of bacteria (Coyne *et al.*, 2004; Gugliandolo *et al.*, 2010; Oliveira *et al.*, 2011). These factors can be extremely important when rapid and accurate detection of pathogenic bacteria is required. In food safety, this is particularly advantageous when risk assessment for human health is required as the first sign of illness often occur in less than 24 h after ingestion of contaminated seafood. Rapid methods are more likely compatible with a faster response to health safety problems (Coyne *et al.*, 2004; Gugliandolo *et al.*, 2010; Oliveira *et al.*, 2011).

In recent years, polymerase chain reaction (PCR) based techniques, has been extensively used in the detection, identification, quantification and characterization of bacterial communities in different samples (Hernández-Zárate & Olmos-Soto, 2006). The use of PCR in combination with the extraction of nucleic acids (DNA and RNA) has been central to the development of culture-independent approaches (Smith & Osborn, 2009). Time-efficient and reliable methods for isolating high-quality nucleic acid from complex food matrices are essential for the success of PCR-based technologies (Coyne *et al.*, 2004).

The PCR-DGGE (polymerase chain reaction – denaturing gradient gel electrophoresis) technique was originally used to detect point mutations in DNA sequences (Cariello *et al.*, 1991; Abrams & Stanton Jr, 1992; Keohavong *et al.*, 1993; Norman *et al.*, 1994) but its use was soon expanded to the assessment of the diversity of microbial communities (Muyzer *et al.*, 1993; Brinkhoff & Muyzer, 1997; Ovreas *et al.*, 1997). This method, mainly based on DNA amplification by PCR assay, generates a genetic profile or "fingerprint" of the microbial community by using the highly conserved domains within the 16S rRNA gene and taking also into account the diversity of microorganisms not cultivable at the present time (Muyzer *et al.*, 1993). Relatively small DNA fragments (commonly up to 400 bp) with the same length but with at least one single base pair difference will be separated, by electrophoresis, in a poliacrylamide gel containing denaturating gradient of urea and formamide, according to the dissimilarity in their melting behaviour (Miller *et al.*, 1999;

Fromin *et al.*, 2002). Double stranded DNA molecule has a specific melting temperature to separate both strains, depending on the hydrogen bonds between the complementary base pairs. During electrophoresis, migration in the increasing denaturing gradient will stop at the first melting domain resulting in a partial separation of the fragment (the complete dissociation of the fragment is prevented by the GC-rich region, GC-clamp, previously added to one primer): DNA segments with low GC content denature at lower denaturant concentration while DNA segments with higher GC content denature further down the gel (Muyzer *et al.*, 1993; Miller *et al.*, 1999; Fromin *et al.*, 2002). DNA fragments with the identical sequences migrate the same distance forming a "band" resulting in a DGGE pattern (Muyzer *et al.*, 1993; Miller *et al.*, 1999). An individual discrete band refers to a unique 'sequence type' which is treated in turn as a discrete bacterial population (Fromin *et al.*, 2002). Further analysis will give qualitative and semi-quantitative results of the most dominant sequence types in the target community (Muyzer *et al.*, 1993; Miller *et al.*, 1999). Despite the importance of this technique, some drawbacks have been recognized, mainly related to the discrimination of the bands (fragments of closely related bacteria are not necessarily resolved or may produce separated bands; nonrelated sequences might co-migrate at an identical position, especially when treating complex community patterns), artificial bands (when analysing complex DNA templates, probably induced by heteroduplex molecules), representativeness of the populations present within a habitat (since they do not all appear on DGGE banding patterns) and reproducibility (Fromin *et al.*, 2002).

4. REDUCTION OF MICROBIAL LOADS: POSSIBILITY OF IMPROVEMENT

Procedures as depuration and relaying have been applied since the 1920s and are extensively used worldwide for the reduction of microbial loads in bivalves. The intent in submitting unhealthy harvested bivalves to natural shellfish beds (relaying) or tanks (depuration) is to enable them to purge from the microbial contaminants by filtering clean water (Richards, 1988; Murchie *et al.*, 2005; Shumway & Rodrick, 2009).

The depuration procedures are primarily dependent on the physiology and ecology of the bivalve (Son & Fleet, 1980; Johnson & Hayasaka, 1988; Richards, 1988; Jones *et al.*, 1991; Murchie *et al.*, 2005) and the availability of growing waters with suitable water

quality in order to be used as a relaying area (Richards, 1988; Lees, 2000). The efficiency of depuration has been based in the same standards used for monitoring growing areas and determined through culture-dependent methods for the detection and quantification of bacterial indicators or pathogens. Notwithstanding the importance of purification methods in reducing, at least partially, the levels of microbial contamination, they are not completely effective in ensuring shellfish safety. There is evidence of the presence of pathogenic autochthonous bacteria (Richards, 1988; Ho & Tam, 2000; Lees, 2000; Croci *et al.*, 2002) and viruses in bivalves which were submitted to purification methods (Son & Fleet, 1980; Romalde *et al.*, 2002; Muniain-Mujika *et al.*, 2003; Marino *et al.*, 2005; Murchie *et al.*, 2005).

Bacteria continue to be related to seafood diseases and are among the most problematic etiological agents. It has been recognized that bacteriophage therapy, meaning the use of lytic bacteriophages (viruses of bacteria) as therapeutic or prophylactic agents, could be an alternative or complementary way of decreasing bacterial loads on organisms and therefore reducing economical loses in bivalve exploitation (Nakai & Park, 2002; Kim *et al.*, 2010; Nakai, 2010; Phumkhachorn & Rattanachaikunsopon, 2010). There is the perspective of using phages to decrease the bacterial contamination in growing areas or as an adjuvant technique of depuration. The application of bacteriophages would improve the safety and quality of the end-product for human consumption.

Aims and structure of the thesis

Microbial contamination of growing beds, and therefore of bivalves, is chronic and pervasive and may be passed on to the consumers. Several preventive measures take into account critical points from harvesting until consumption in order to ensure public health safety. Nevertheless, microbial contamination of bivalves is underestimated and undermanaged, imposing potential risks to public health. This work indents to approach relevant points in the improvement of the safety of bivalves to the consumer. For that purpose the following specific objectives were established for this thesis:

- To characterize the development of Portuguese bivalve exploitation highlighting critical points requiring improvement in this sector of activity (**Chapter I**)
- To outline crucial aspects in monitoring of growing areas that could be improved (**Chapter II**)
- To develop a culture-independent method suitable for the analysis of the whole organism as well as the surrounding environment, that can be useful in monitoring bivalve sanitary quality (**Chapter III**)
- To discuss an alternative for reducing the microbial contamination and developing the purification of bivalve shellfish, namely by delineating the future perspective of bacteriophage therapy (**Chapter IV**).

The present thesis begins with a general introduction to contextualize the aims of the work and finalizes with a section for general conclusion in order to systematize and resume the results obtained and outline major conclusions and perspectives. The remaining sections are structured in chapters, which correspondingly incorporate most of the specific objectives described above. In each chapter, independent subchapters address different issues related to the main topic.

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Chapter I

Development of the Portuguese bivalve harvesting and production – an overview

Bivalve harvesting and production in Portugal – an Overview

In: Oliveira, J., Castilho, F., Cunha, A. & Pereira, M.J. (submitted). Bivalve harvesting and production in Portugal – an overview.

ABSTRACT

The exploitation of bivalves is an ancient activity in Portugal, with social, economic and cultural importance. The profits associated to the exploitation of molluscs are largely dependent on wild captures of bivalves. Nevertheless, the production of bivalves has a relevant impact on the aquaculture sector. The latest data indicates a profit of 57 858 million euros (22.7% of the profit from capture in 2010) and 23 695 million euros (53.5% of the profit from aquaculture in 2009) related to harvesting and production, respectively. Clams, cockles, oysters, razor shells and mussels are the main harvested and produced species. Molluscs are mainly produced under the extensive regime and destined to the national market. Nevertheless, Japanese oysters were entirely exported while mussels were channeled for both markets. In 2009, the volume of business has grown with the international trade but higher imports caused a loss of 110 723 million euros. There is considerable potential for expansion of the bivalve exploitation sector and industry in Portugal. New strategies to overcome constraints to its development are urgently needed, namely: (1) improving the communication among stakeholders, (2) to encourage the organization of the sector and (3) to add value to the product creating new market opportunities.

KEYWORDS

Shellfisheries; Bivalve production; Shellfish economy; Shellfish quality; Portugal.

1. INTRODUCTION

Bivalve molluscs represent significant proportion of the world fishery exploitation (FAO, 2010b). In Portugal, the harvesting and production of bivalves are ancient activities. They are practiced from north to south of Portugal with significant impact in the national economy. It is worth of notice that Tagus estuary harbors the largest natural oyster bed of Europe (IPIMAR, 2008).

The exploitation of bivalves is considered highly attractive by various reasons. There is a long tradition of artisanal cultivation and most techniques for raising bivalves are simple and readily adaptable to most parts of the Portuguese coast and even to other parts of the world (Rice, 1992). Although molluscs have been exploited for hundreds of years, recent advances in cultivation technology have led to a significantly increase of production (Helm & Bourne, 2004). Since, bivalve molluscs are filter-feeders which depend on the natural primary productivity at the cultivation site, feeding costs are not implied (Rice, 1992; Helm & Bourne, 2004). Also, they generally require minimum husbandry (Helm & Bourne, 2004) and have a wide range of temperature tolerance (5ºC to 35ºC) and often, somatic growth doubles or triples with each 10ºC increase in ambient temperature (Rice, 1992).

Harvesting and production activities are aimed at obtaining high quantities of safe and high-quality products for human consumption. Safety usually refers to the level of risk associated with illness or death caused by the ingestion of a seafood product that is contaminated (Cato, 1998; Oliveira *et al.*, 2011). Quality is most often related to the appearance, odor, flavor and texture but it includes also nutritional value, shelf life, level of additives, presence of shell imperfections, size and uniformity, presence of food preservatives (Cato, 1998). Obtaining a product with these characteristics is demanding and entails costs.

The improvement of the global system of production and harvesting of shellfish, with the implementation of some strategies at different levels in this sector (government agencies, producer/fisher, traders and consumers), could lead to a more profitable and sustainable development. We aimed to provide the current state of harvesting and production activity of bivalves in Portugal pointing some important measurements for their further development.

2. BIVALVES IN PORTUGUESE FEEDING HABITS

Portugal has one of the world highest *per capita* consumption of seafood products (about 58.5 kg/person/year), closely following the leaders Japan and Iceland (Paquotte & Lem, 2008). Bivalves are consumed and appreciated for a variety of reasons: food preferences (minimally processed and additive-free), tradition, nutritional value, changing lifestyles, economics, food availability, population growth (Cato, 1998; Fauconneau, 2002; Murchie *et al.*, 2005).

In some countries, religion may also play an important part in feeding habits. For instance, dietary laws of Seventh-day Adventists, Jewish, Muslims (Hanafi) and the Shi'ites (Ja'fari) forbid the consumption of shellfish. However, in Portugal, 84.5% of the Portuguese population is Roman Catholic, followed by 9.0% of the population that self-declare non-religious (INE, 2001). This means that the predominant religion has no dietary restrictions in respect to shellfish and does not greatly influence the Portuguese consumption of these animals. Bivalve molluscs, either fresh or processed, play an increasing role in the Portuguese diet (Fonseca *et al.*, 2006). Being characteristically tender, easily digested, additive-free and minimally processed, fresh shellfish are highly required by Portuguese consumers and by tourists (Fonseca *et al.*, 2006). Shellfish is usually eaten raw or slightly cooked and with little or no garnishing. However, some of the most appreciated Portuguese dishes usually combine fish, shellfish and meat together, namely: *Arroz de marisco* (seafood rice), *Caldeirada, Amêijoas à bulhão pato*, *Cataplana* and *Carne de porco à alentejana* (Rosa-Limpo *et al.*, 1946).

Bivalves have great nutritional interest and the benefits of seafood have been recognized. The nutritional value of bivalve molluscs is primarily dependent on the quality of the aquatic environment from which they originate, which assures both a healthy and safe product for consumption. Water temperature, nutrient availability and reproductive cycle of animals may also influence biochemical composition and meat quality of some bivalves (Karakoltsidis *et al.*, 1995; Orban *et al.*, 2002). Nutritional value varies among bivalve species. Protein content is similar to that of milk and eggs, being an important source high-quality animal protein and composing approximately 13.0% in most Portuguese bivalve species (15.0% in common cockles and 17.0% in scallops) (Bandarra *et al.*, 2004; IPIMAR, 2008; Oliveira *et al.*, 2011). Bivalves might almost completely fulfill the balanced demand for essential amino acids in adult consumers (Fauconneau, 2002). This is

interesting since Portugal is a developed country with an increasing growth of the elderly population (INE, 2011). Carbohydrate content varies throughout the year according to the reproductive cycle of bivalves but it is usually largely constituted by glycogen. Fat content is low (usually, not exceeding 3.0%) suitable for a healthy diet, with the amount of unsaturated fatty acids (particularly cholesterol-reducing Omega-3 fatty acids) being higher than the amount of saturated fatty acids. Finally, Portuguese seafood has high content of vitamins A, D, E and B12 and minerals (Cato, 1998; Bandarra *et al.*, 2004; IPIMAR, 2008). Being rich in proteins, vitamins and having low-fat content, seafood consumption can help to prevent the ingestion of excessive calories, cholesterol, total and saturated fat providing consumers with a healthy alternative (Cato, 1998).

3. HARVESTING AND PRODUCTION AREAS

Portugal is located in southern Europe with an area of 92 090 km^2. It is the western-most country of continental Europe and occupies the Atlantic front of the Iberian Peninsula being bordered by the Atlantic Ocean to its west and Spain to the north (Figure 1) (FAO, 1993). Due to its geography, Portugal combines a series of characteristics that can be considered ideal for the development of the bivalve production sector: (1) an extended Exclusive Economic Zone with more than 3 877 408 km^2 (Anonymous, 2009), (2) 1793 km of coast under the influence of different currents, (3) Mediterranean climate with Csa in the south and Csb in the north, according to the climatic classification of Köppen-Geiger (Bebianno, 1995; Instituto de Meteorologia, Last accessed 23 November 2011), (4) water temperatures varying between 13ºC and 18ºC, according to latitude (higher values are expected in the south, while lower ones are expected in the north cost), (5) estuaries and lagoons from north to south and (6) high primary production (FAO, 1993; Machás *et al.*, 2003).

Bivalve molluscs have two different origins: (1) catching of wild animals in open-sea coastal waters along the Portuguese coast, as well as in inshore banks and natural ponds; (2) production in the estuarine waters (Paquotte & Lem, 2008). Shellfish farming in Portugal is based on two types of production, inshore and offshore. Both involve the capture of seeds with larvae collectors and subsequent placement in nurseries (IPIMAR, 2008).

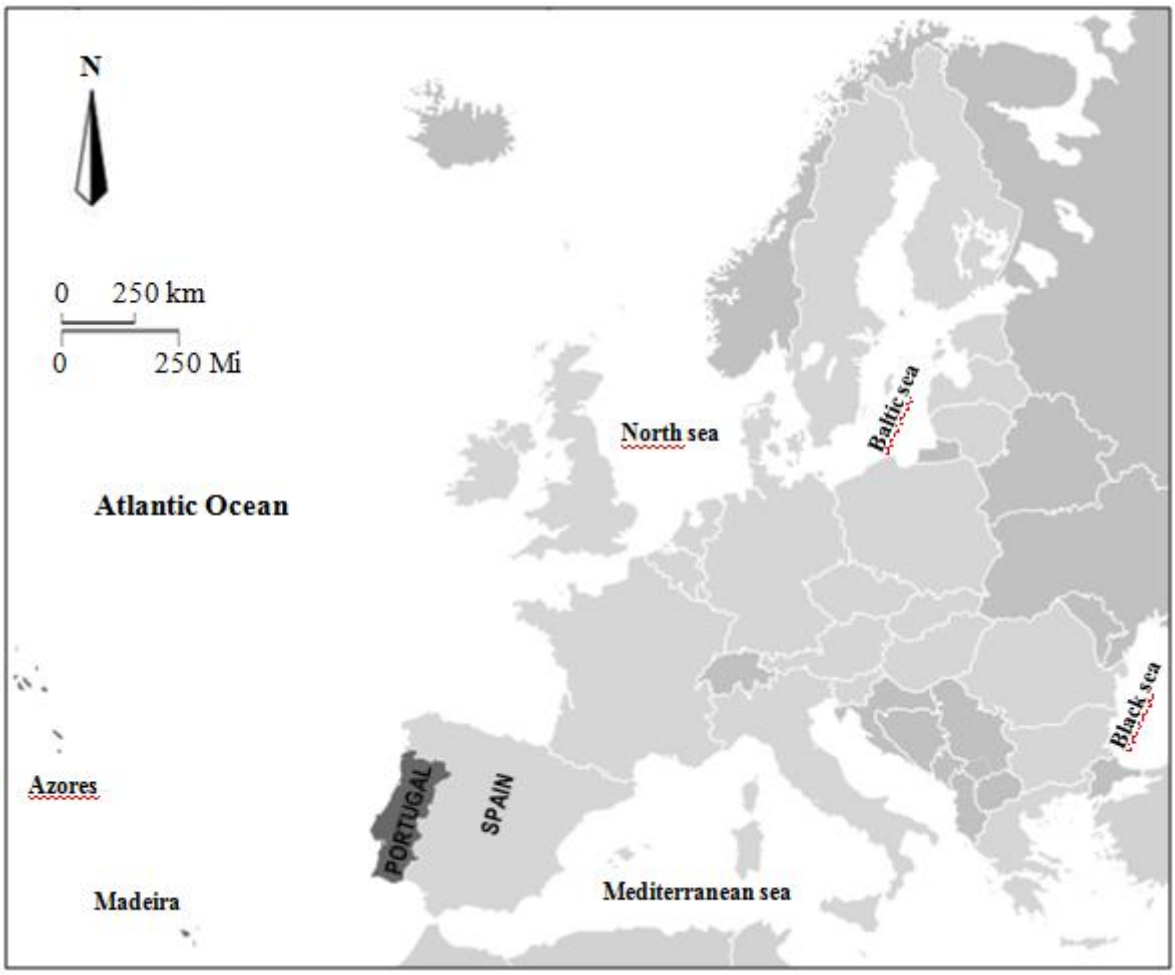

Figure 1 Portugal location in Europe. Portugal is shown in dark-grey. European Union is represented in light grey. Territories not geographically in Europe are shown in a medium shade of grey (Adapted from Wikipedia, Last accessed 23 August 2010).

The quality of the shellfish harvesting and production areas depends on the geographic characteristics of the coast and the coexistence with other human activities (agricultural, urban, industrial, livestock, among others) that generate pollution and strongly influence water quality (Laing & Spencer, 1997; IPIMAR, 2008). The fact that these organisms filter large volumes of water to obtain oxygen and nutrients and, consequently, became vectors of various chemical and biological contaminants (Oliveira *et al.*, 2011), implies an ongoing effort by several entities, to ensure the quality and safety of shellfish. The classification of harvesting and production areas according to their risk in relation to food safety, allows the some anticipation on the microbiological quality of shellfish caught in those areas. Accordingly, have implications on the type of treatment that shellfish must be submitted

prior to marketing, thereby helping to reduce risks associated to public health. All shellfish intended for human consumption must meet the specific microbiological criteria set out in Annex I of the Regulation (EC) Nº 2073/2005 (Anonymous, 2005) and Regulation (EC) Nº 1441/2007 (Anonymous, 2007) and also the quality parameters defined in Chapter V, Section VII, Annex III Regulation (EC) Nº 853/2004 (Anonymous, 2004a), Annex II, Chapter II Regulation (EC) Nº 854/2004 (Anonymous, 2004b) and Regulation (EC) Nº 1021/2008 (Anonymous, 2008b), and the content of toxic metals specified in Regulations (EC) Nº 1881/1006 (Anonymous, 2006a) and Nº 629/2008 (Anonymous, 2008a). Therefore, the monitoring of shellfish harvesting and production areas is essential and it demands a well designed sampling program and the clear definition of the production zones.

The state laboratory of the National Institute of the Biological Resources/ Fisheries and Sea Research Institute (INRB, I. P./IPIMAR) of the Ministry of Agriculture, Rural Development and Fisheries, is the authority responsible for (1) the sanitary control of bivalves for human consumption, (2) the technical and scientific advice regarding installation of new bottom culture beds and (3) the classification and monitoring of the harvesting and production areas, according to the health standards for the production and marketing of live bivalve molluscs for direct human consumption (Decree-Law Nº 112/95, N º 236/98 and Decree Nº 1421/2006) (Campos & Cachola, 2006). According to Order No. 14515/2010 of the President INRB, I. P. of 17 July 2010 (Republic Diary Nº 182, Series II), Portuguese production areas are defined in 17 estuarine zones and 9 coastal areas (Figure 2) (IPIMAR, 2008). The areas that register the higher production of bivalves are the center of Portugal and the Algarve (southern Portugal), together accounting for 72.7% of the national production. These regions, display the most suitable adequate physical conditions for bivalve harvesting (DGPA/INE, 2011) which is usually carried out as a complement of other economic activities, either by fisher or by other agents, directly involving 8000-10 000 people only in the Algarve (Campos & Cachola, 2006).

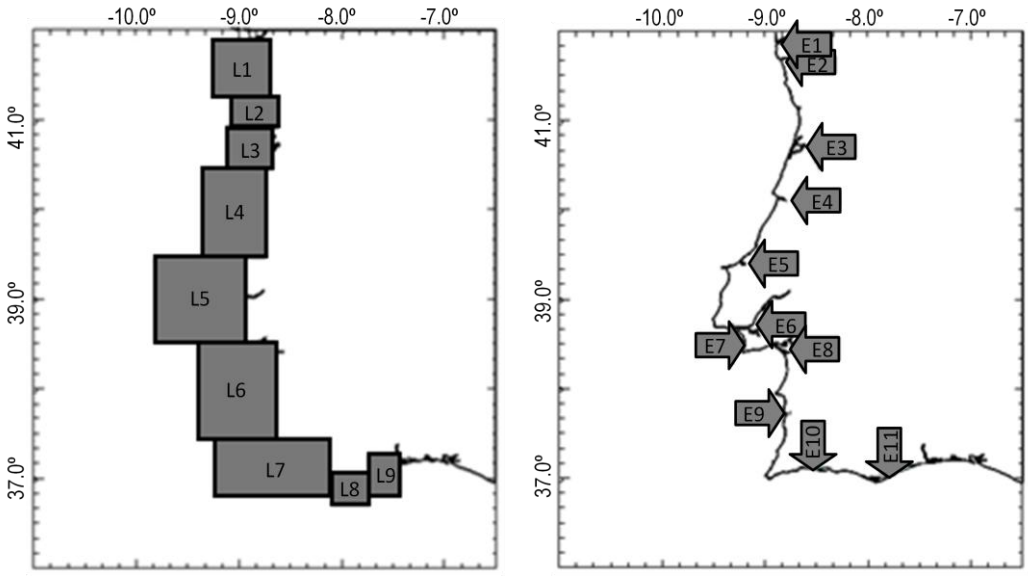

Figure 2 Portuguese definition of the bivalve's production zones (Adapted from IPIMAR, 2008). In the figure: L- Coastal production zone (map on the left); L1- Viana; L2- Matosinhos; L3- Aveiro; L4- Nazaré and Figueira da Foz; L5- Lisboa and Peniche; L6- Sines and Setúbal; L7- Portimão and Lagos; L8- Olhão and Faro; L9- Vila Real de Santo António and Tavira; E- Estuarine production zone (map on the right); E1- Estuário do Lima and Estuário do Minho (2 zones); E2- Estuário do Douro; E3- Ria de Aveiro; E4- Estuário do Mondego; E5- Lagoa de Óbidos; E6- Estuário do Tejo; E7- Lagoa de Albufeira; E8- Estuário do Sado; E9- Estuário de Mira; E10- Rio do Alvor and Rio Arade (2 zones); E11- Ria Formosa (5 zones).

4. HARVESTING VALUES OF BIVALVE SHELLFISH IN PORTUGAL

From 1999 to 2006, Portuguese captures of both fish and molluscs decreased 16.8% because of the high dependence on fish captures, which decreased 18.8%. Notwithstanding, mollusc captures registered a growth of 11.6% in the same period (Figure 3). In the two following years, fish captures increased to 148 387 tons and mollusc captures reached 20 341 tons. In 2009, both fish and mollusc captures decreased to 126 479 tons and 16 147 tons, respectively. In 2010, captured fish totalized 145 767 tons, representing 197 246 million euros (an annual growth of 17.0%) while captured molluscs totalized 18 885 tons, with an estimated value of 57 858 million euros (an annual growth of 15.2%). Still considering the 1999–2010 period, the highest values of both fish and mollusc captures were registered during in 1999 and 2008 totalizing 152 868 tons and 20 341 tons, respectively. Overall balance of the variation during this decade corresponds to a reduction in the captures of mollusc and fish of 2.4%, mainly due to fish captures which

largely exceed (by about eight times) mollusc captures. Actually, mollusc captures grew at a rate of 4.0% per year. The generalized decrease in captures reflect the efficiency of the control measures related to the need of preserving stocks of natural resources and the effect of restriction periods imposed to the capture of different species, and ultimately a response of the authorities to the need for a sustainable development of fisheries. During this decade, fish accounted for approximately 87.9% of the captures in both brackish and marine waters, representing 73.0% of the economic profit. In the same period, molluscs accounted for 11.1% of the captures (20.4% of the economic profit) while the remaining 1.0% were due to capture of crustaceans and other aquatic products.

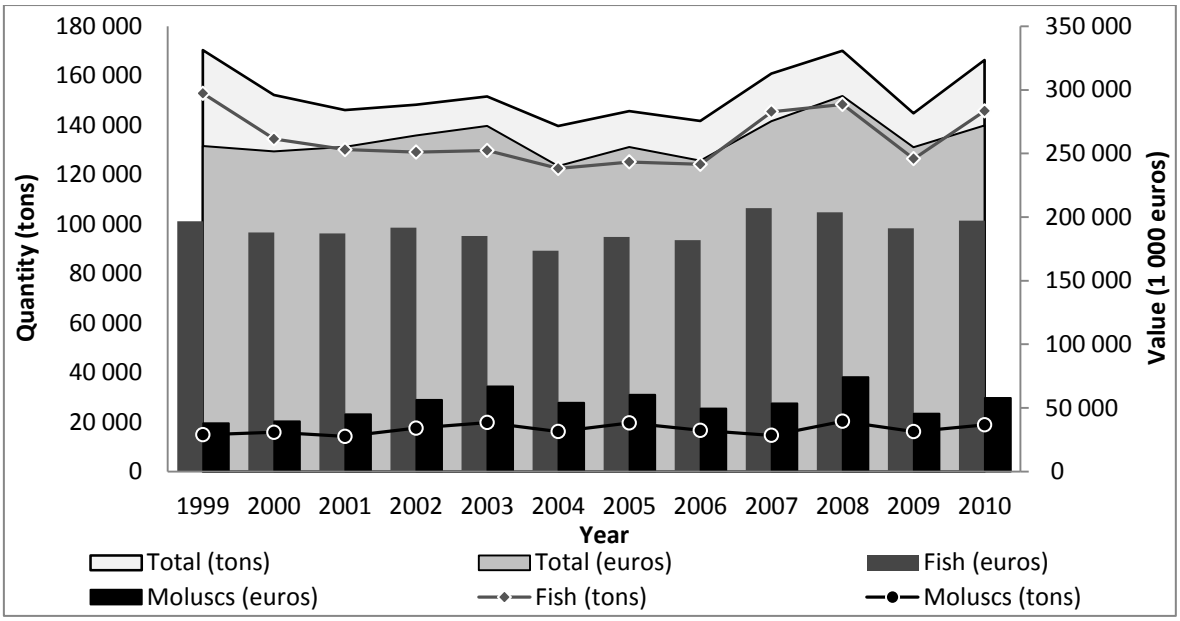

Figure 3 Portuguese harvesting values (in quantity and value) for the two major captured groups (fish and molluscs) between 1999 and 2010 (DGPA/INE, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011). Data related to the quantity of captured fish and molluscs (tons) for 1999 are not available.

Several species of bivalves are commercialized in Portugal (Anonymous, 2006b, c; Garibaldi & Busilacchi, 2010). The main harvested bivalves were cockles, clams, razor shells, mussels and oysters (Table 1, Figure 4). The harvesting of different types of shellfish fluctuated erratically over this last decade but it is clear that cockles and clams remain the most captured bivalves, followed by razor shells, mussels, and finally oysters

(Figure 4). In 2010, cockles represented 14.8% of the national shellfish capture accounting for 2185 million euros and clams represented 4.8% corresponding to a profit of 2996 million euros were among the most produced species; razor shells, oysters and mussels, came next in quantity with 0.6% (299 thousand euros), 0.3% (45 thousand euros) and 0.2% (44 thousand euros), respectively (DGPA/INE, 2011).

Table 1 List of species of bivalve molluscs according to their interest to fisheries in Portugal (data from 2009).

Scientific name	Author	English name	3-alpha	ISSCAAP
Cerastoderma edule	(Linnaeus, 1758)	Common edible cockle	COC	56
Cerastoderma glaucum	(Bruguiere, 1789)	Olive green cockle	KTG	56
Crassostrea angulata	(Lamark, 1819)	Portuguese oyster	OYP	53
Crassostrea gigas	(Thunberg, 1793)	Pacific cupped oyster, Japanese oyster	OYG	53
Ensis ensis	(Linnaeus, 1758)	Pod razor shell	EQE	56
Ensis siliqua	(Linnaeus, 1758)	Sword razor shell	EQI	56
Mytilus edulis	(Linnaeus, 1758)	Blue mussel	MUS	54
Mytilus galloprovincialis	(Lamarck, 1819)	Mediterranean mussel	MSM	54
Ostrea edulis	(Linnaeus, 1758)	European flat oyster	OYF	53
Pharus legumen	(Linnaeus, 1758)	Bean solen	FRL	56
Ruditapes decussatus	(Linnaeus, 1758)	Grooved carpet shell	CTG	56
Ruditapes philippinarum	(Adams & Reeve, 1850)	Japanese carpet shell	CLJ	56
Solen marginatus	(Pulteney, 1799)	European razor clam	RAE	56
Spisula solida	(Linnaeus, 1758)	Solid surf clam	ULO	56
Venerupis pullastra	(Montagu, 1803)	Pullet carpet shell	CTS	56

In the table: ISSCAAP - code assigned according to the FAO "International Standard Statistical Classification for Aquatic Animals and Plants" (ISSCAAP) which divides commercial species on the basis of their taxonomic, ecological and economic characteristics into the group of molluscs and the division of clams, cockles and arkshells (56), mussels (54) and oysters (53); 3-alpha - a unique code made of three letters that is widely used for the exchange of data with national correspondents and among fishery agencies; nei- not elsewhere included (Adapted from Anonymous, 2006b, c; Garibaldi & Busilacchi, 2010).

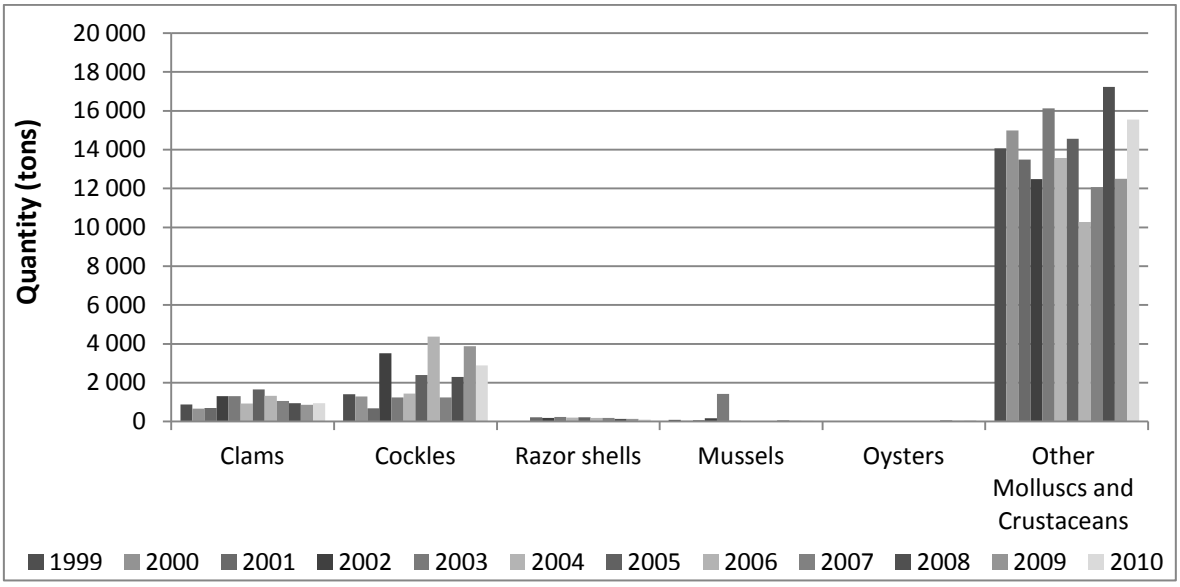

Figure 4 Portuguese bivalve harvesting values (in quantity per year) between 1999 and 2009 (DGPA/INE, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011).

4.1. TRADE OF CAPTURED BIVALVES

In the last decade, Portuguese imports of live, fresh, chilled or frozen molluscs largely exceed exports. Also, a gradual increase of both imports and exports was observed until 2009. In this year, the imports of these molluscs reached 63 118 tons, representing 159 373 million euros. Imports come mainly from Spain, France, Netherlands, Switzerland, India, Mexico and China. In 2009, exports to international markets such as Spain, Italy, France, the USA, Switzerland and Angola, fulfilled 17 370 tons, representing a profit of 55 615 million euros (Figure 5). In 2010, these exports recorded a strong increase totalizing 123 923 million euros (17.5% of total output) (DGPA/INE, 2011). Despite the exportation increase, the balance of the international trade of captured molluscs was still negative, with a loss of 56 287 million euros (DGPA/INE, 2011).

The market of canned mollucs was not very expressive when compared to the trade of live, fresh, chilled or frozen molluscs (Figure 5). In 2010, the value spent with live, fresh, chilled or frozen molluscs imports was 12 times higher than the cost related to canned products (14 578 million euros). In the same year, canned products represented a profit of 8662 million euros, an income 14 times lower than the resulting profit of live, fresh, chilled or frozen molluscs exports (Figure 5). During the last decade, imports of canned products largely exceed exports (Figure 5). Recently, the canning industry has relied on exports for

minimizing the existing negative balance. Indeed there is an expected trend of the market of canned molluscs to increase in the face of their convenience of use (IPIMAR, 2008).

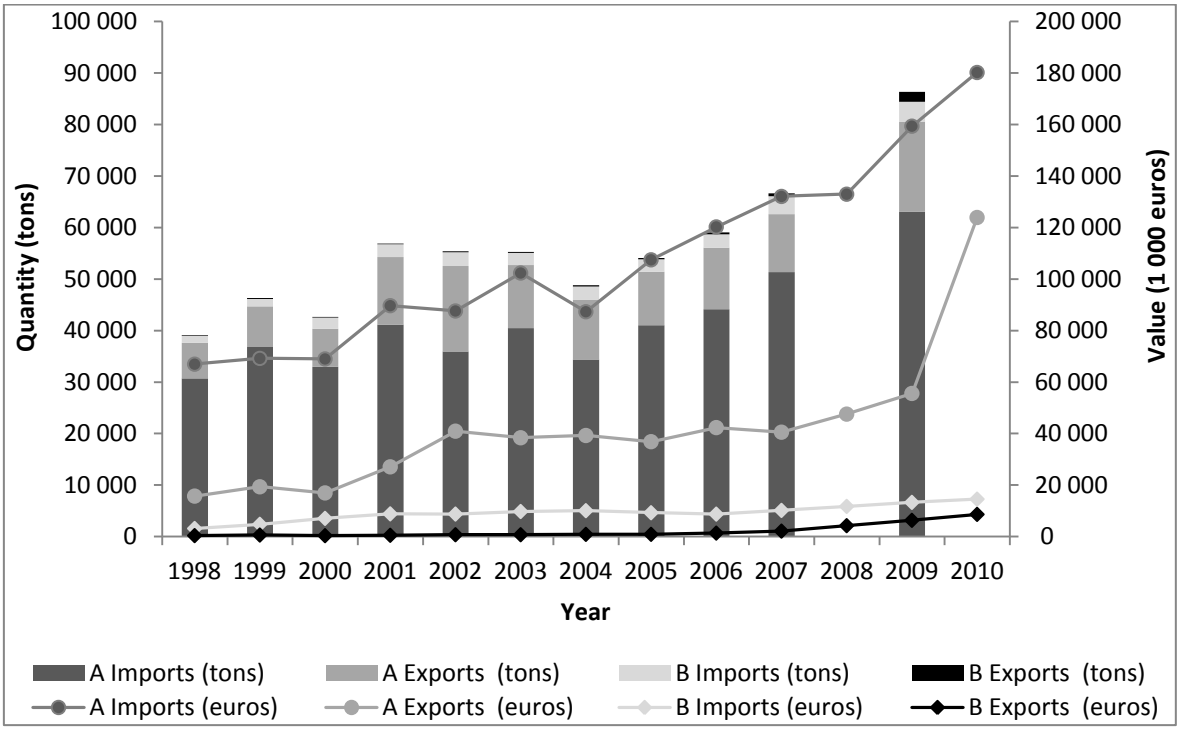

Figure 5 Exports and imports (in quantity and value) of live, fresh, chilled or frozen molluscs and other aquatic invertebrates (A) and of canned molluscs and crustaceans (B), from 1998 until 2010 (DGPA/INE, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011). Data related to the quantity of imports and exports (tons) for 2008 and 2010 are not available.

5. PRODUCTION VALUES OF BIVALVE SHELLFISH IN PORTUGAL

In Portugal, in the 1990–2009 period, the Portuguese aquaculture sector grew 79.3% (Figure 6), but the average annual growth rate is still very low (4.2%) in relation to the rest of the world, where the average annual growth rate is 6.6% (FAO, 2010a). This increase in the production reflects infrastructural improvements, wider use of proper equipment, greater availability of juveniles, the introduction of more production systems and the increase in the number of aquaculture units (Bernardino, 2000). Until 1998, mollusc cultivation exceeded fish cultivation. However, between 1995 and 2000, fish production increased to 4168 tons becoming the dominant aquaculture product. In the last years, fish production remains the main aquaculture product but with an average increase of only

2.9% per year. Mollusc production shows a tendency to strongly fluctuate over time. Abrupt drops in mollusc production might be related to: (1) the number of sites available (which may not in itself be a limiting factor, although all sites must have or obtain a classification) (Laing & Spencer, 1997); (2) progressive deterioration of the water quality (lacking shellfish growing water with acceptable sanitary quality) (Bernardino, 2000); (3) the reduction of natural shellfish beds as a consequence of over-catching (Pillay & Kutty, 2005); (4) the relative abundance of many of the main commercial species along the Portuguese coast (Anonymous, 2010); (5) a shrinking fleet with poorly trained professionals (Anonymous, 2010); (6) the variability of mortality associated with bivalve handling (Laing & Spencer, 1997); (7) the inadequacy of bivalve seeds (Bernardino, 2000); (8) the mortality caused by predators (Laing & Spencer, 1997). As the aquaculture sector is largely dependent on mollusc cultivation, the total values followed their tendency.

In the year 2009, aquaculture production was 7992 tons, representing 44 262 million euros in value. Shellfish accounted for approximately 48.2% (3851 tons) of production in brackish and marine waters, representing 53.5% of the economic profit obtained from aquaculture (DGPA/INE, 2011). Over the last decade, despite the fluctuations and having lower values of production than fish aquaculture, molluscs accounted for the highest economic profit of the aquaculture products (Figure 6).

Within the national context, the main produced bivalve molluscs were clams, oysters, mussels and cockles (Figure 7). The production of different types of shellfish did not change significantly over this last decade: the clams have the lead in production, followed by oysters, mussels, and cockles (Figure 7). In 2009, clams represented 60.9% of the national shellfish aquaculture accounting for 22 191 million euros; oysters represented 24.7% corresponding to a profit of 1179 million euros and the Japanese oyster was among the most produced species (12.0%); mussels and cockles came next in quantity with 7.9% (163 thousand euros) and 6.5% (152 thousand euros), respectively (DGPA/INE, 2011).

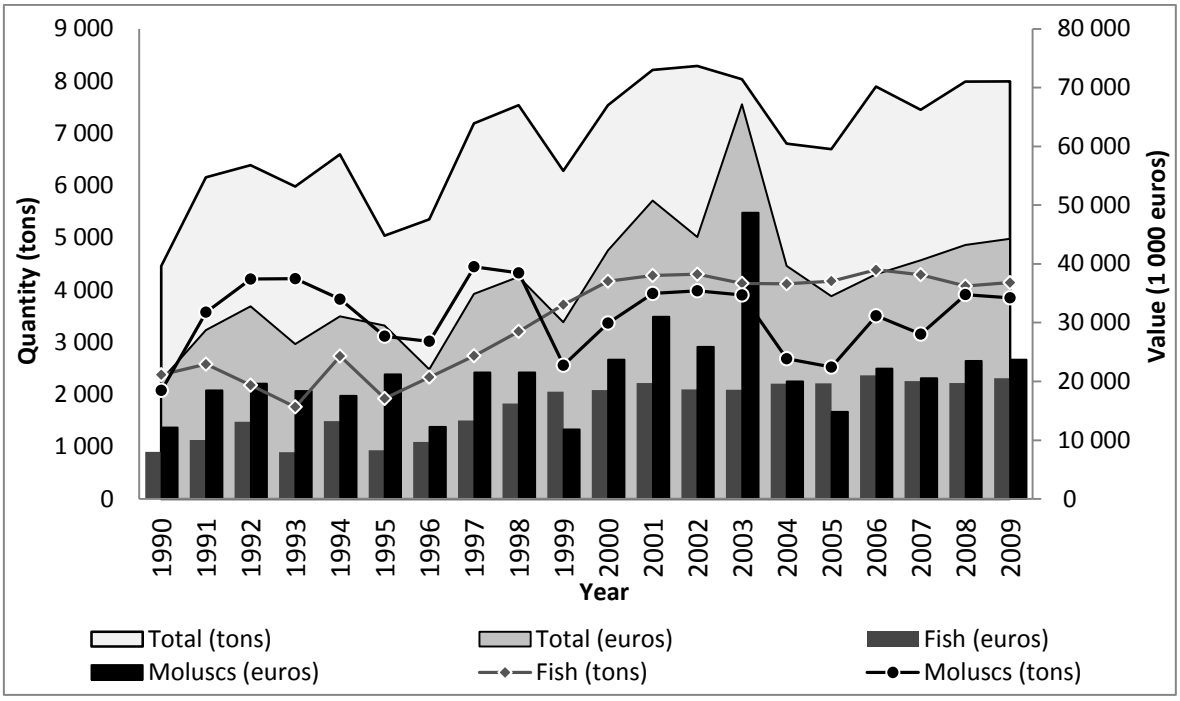

Figure 6 Portuguese production values (in quantity and value per year) for the two major aquaculture groups (fish and molluscs) between 1990 and 2009 (DGPA/INE, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, unpublished data).

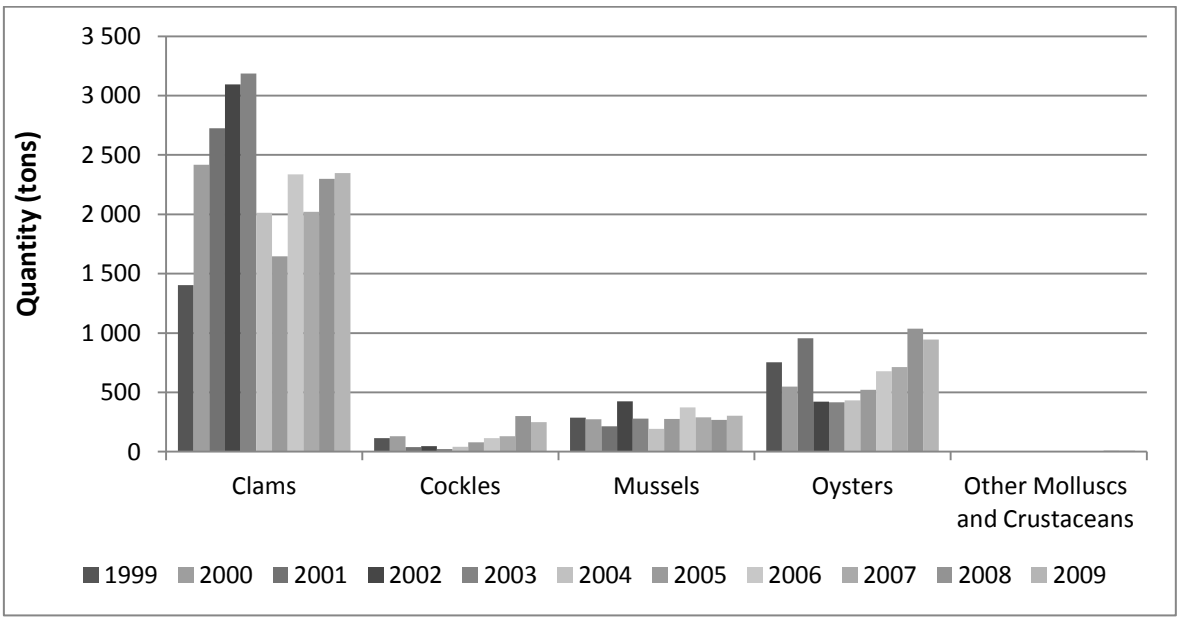

Figure 7 Portuguese production values (in quantity per year) for the aquaculture bivalves between 1999 and 2009 (DGPA/INE, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, unpublished data).

5.1. PRODUCTION SYSTEMS

In 2009 there were 1525 establishments in aquaculture, from which 1489 were licensed establishments for salt and brackish water. Of these, 89.8% were the nurseries for the production of bivalve molluscs, most of which located in the *Ria Formosa* (DGPA/INE, 2011). Between 1999 and 2000, the number of units operating extensive regime largely increased reaching 1301 units (Figure 8). Since 2000, a slow but progressive increase has been registered in the number of establishments operating extensive regime. During the period of 1999 to 2009, the number of units operating intensive and semi-intensive regimes increased slightly. In 2009, a total of 1374 establishments operated extensive regime, 57 semi-intensive and 12 intensive (DGPA/INE, 2011). The extensive regime dominates mollusc cultivation. During this decade, the semi-intensive system represented a small contribution to global production of molluscs, never exceeding the 341 tons obtained in 2006 (Figure 8). Production of molluscs by the intensive regime only occurred in 2009 accounting for 30 tons while semi-intensive regime summed 140 tons (Figure 8).

A business of cultivation of bivalves is dependent on a regular annual supply of juveniles, known as seed or spat, for growing on to market size. Seed might be obtained locally on natural beds. However, when this supply is not sufficient, seeds are collected from distant areas or acquired from national or international facilities involving expensive transport costs (Laing & Spencer, 1997). In 2009, a total 10 082 millions of juveniles were used in the Portuguese cultivation of molluscs and crustaceans. Most juveniles used in mussel production come from wild catch (2635 millions) or national facilities (244 thousand). On the other hand, 7200 million juveniles of the Japanese oyster come from international facilities. European and Portuguese oysters accounted for 2 thousand juveniles each, caught in the natural environment.

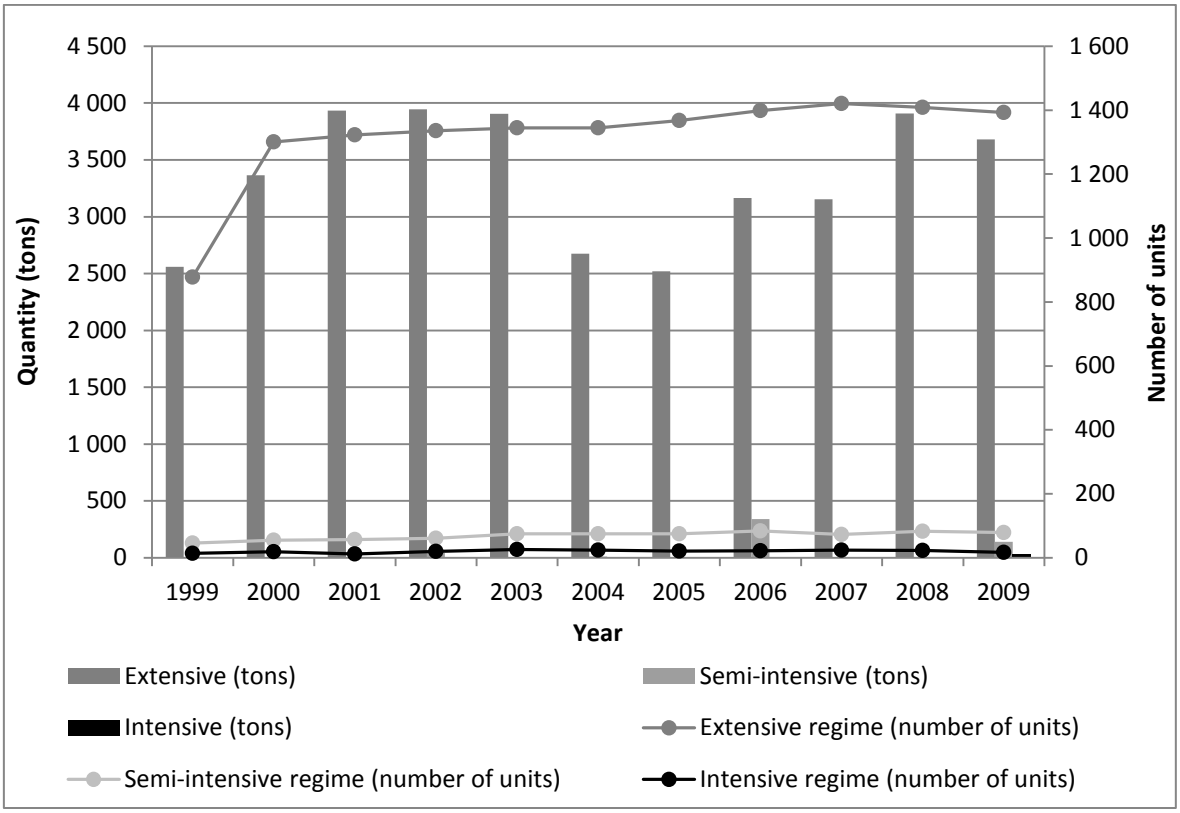

Figure 8 Number of units by production regime in salt and brackish water from 1999 until 2009 and respective bivalve production (in quantity by year) (DGPA/INE, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011). In the figure: Extensive - species reared on natural productivity of the pond/area; Semi-Intensive - species reared on natural productivity but with the addition of some artificial diets; Intensive - species reared solely on artificial diets.

5.2. TRADE OF PRODUCED BIVALVES

During the period of 2005–2009, the highest profit (89.4%) from aquaculture products was obtained from national market while the remainder (10.6%) came from the international transactions (Figure 9). Clams and cockles were exclusively commercialized in the national market while the production of the Japanese oyster was entirely exported (Figure 9). In 2009, clams was the most sold mollusc (2340 tons), representing a total profit of 22 162 million euros (DGPA/INE, 2011). In 2009, 162 tons of Japanese oysters were sold, representing a value of 292 thousand euros (DGPA/INE, 2011). The production of mussels was channeled for both the national and international markets (Figure 9). Most of the bivalve production was exported to western European countries, especially France and Spain (Campos & Cachola, 2006). In Portugal, bivalves were sold in local markets and

were mainly consumed fresh by resident population and tourists in restaurants and seafood festivals (Campos & Cachola, 2006). The Algarve tourism industry is now emphasizing gastronomic tourism as an important complement for the sustainability of the bivalve production sector (Campos & Cachola, 2006).

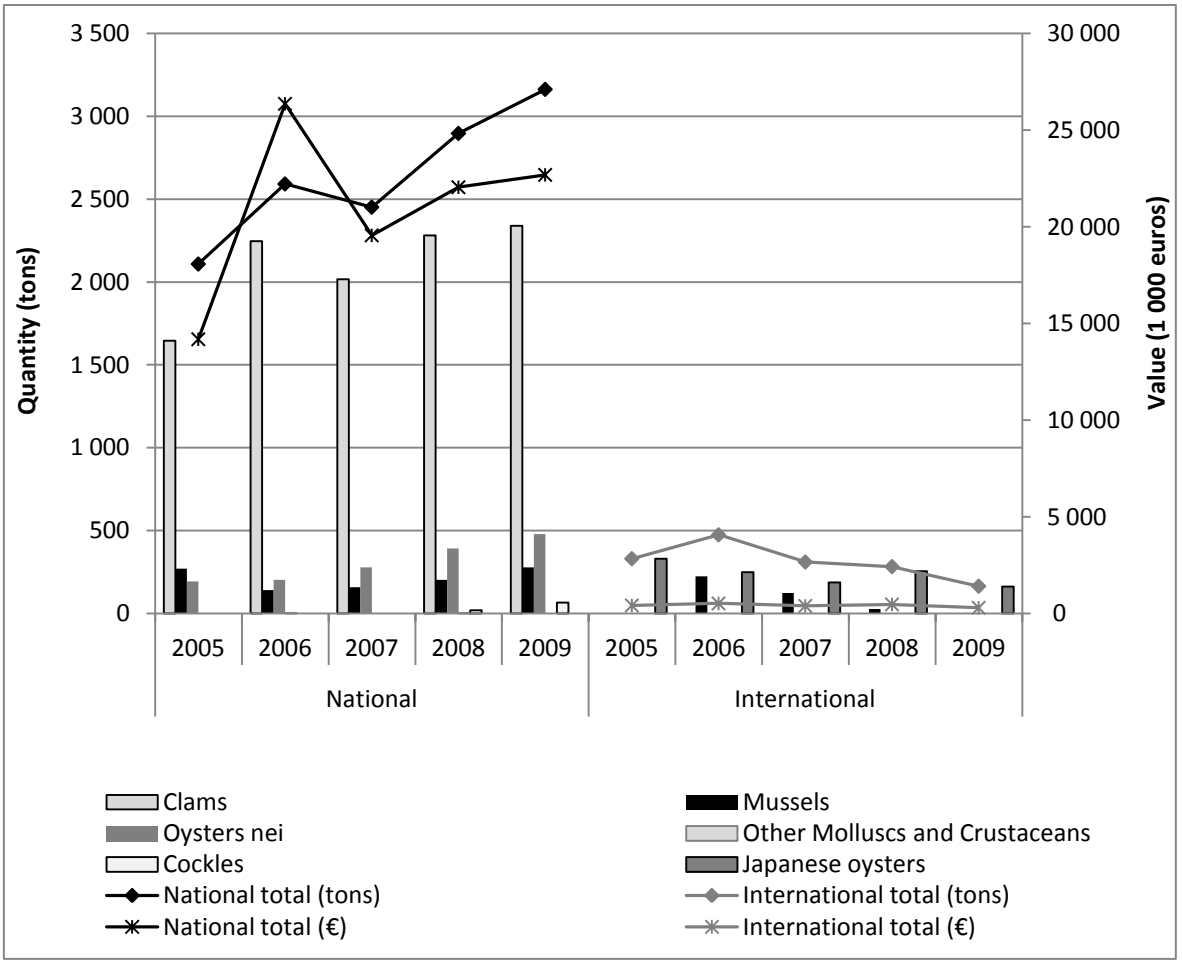

Figure 9 Sale values of bivalve molluscs (in quantity per year), by major commercialized bivalves, for the national and international market between 2005 and 2009 (DGPA/INE, 2007, 2008, 2009, 2010, 2011). Data from 1999 to 2004 are not available.

6. STATE OF THE EXPLOITATION OF THE BIVALVE MOLLUSCS

The wild capture represents the major supply for mollusc market, almost reaching 20 500 tons in the last decade. Mollusc capture value is nearly five times higher than that

corresponding to mollusc production which, in the last years, never surpassed 5000 tons (Figures 4 and 9).

In 2009 and 2010, bivalve capture value summed 45 540 million euros and 57 858 million euros, respectively representing about 20% of the economic profit obtained from capture of both molluscs and fish in each of these years. On the other hand, bivalve production yielded 23 695 million euros in 2009, more than half of the economic profit obtained from aquaculture products. Although production of bivalves is fairly represented in the aquaculture sector, incomes of mollusc sales were mostly obtained from the wild capture. It is important to notice that these values might be underestimated because they are supplied by fishers who tend to undervalue the quantities harvested or cultured as well as their profits (Bernardino, 2000; IPIMAR, 2008), and therefore, the global profit associated with mollusc production and trade might be underestimated.

Mollusc cultivation, mainly by the extensive regime, was dominated by clams, oysters, mussels, and cockles. The main harvested bivalves by decreasing order of quantity of capture, were cockles, clams, razor shells, mussels and oysters. Mussels and oysters were channeled for both national and international markets.

In 2009, mollusc exploitation in Portugal (capture and production) totalized 19 998 tons. In the same year, 17 370 tons of live, fresh, chilled or frozen molluscs were sent to the international market and imports of similar products summed 63 118 tons. Also, 3909 tons of canned molluscs and crustaceans were imported and 1960 tons exported. The increased imports of foreign mollusc products (live, fresh, chilled or frozen molluscs and canned molluscs and crustaceans) lead to negative balance of 110 723 million euros.

Bivalve consumption and the demands of consumers may explain the large amount of imports. Nevertheless, these values along with other costs related to bivalve exploitation, show that the sector of bivalve exploitation needs new and prompt strategies to invert this trend.

7. BARRIERS HAMPERING THE DEVELOPMENT OF THE EXPLOITATION OF THE BIVALVE MOLLUSCS AND PROPOSED STRATEGIES TO CIRCUMVENT THEM

The exploitation of marine resources has been a mainstay for coastal communities. Initially, it was a balanced and sustainable activity, practiced mainly at a subsistence level

and as a supplementary income. However, it has currently reached a dramatic overexploitation due to the sharp increase in demands associated to population growths. The (1) increasing need for coastal areas for leisure and tourism and the consequent increase in the exploration effort, (2) the rapid improvement of capture technologies, (3) the adjustment of the laws regulating the sector, and (4) the progressive reorganization of the fishing sector have contributed to the increase of pressure upon marine resources, resulting in a higher exploitation of the biomass with positive economic and social consequences.

Several barriers limit, to a greater or slighter degree, the possibilities of developing the harvesting and production of shellfish: (1) training of fishers and fishfarmers, (2) the transference of technology to the interested parties in the sector, (3) the organization of the sector, (4) the competitiveness of prices, (5) the monitoring of the products and of the productive process, (6) the sanitary certification of breeding areas and mollusc products, (7) the acquisition or assignment of suitable coastal areas and their legal allocation to production, (8) the establishment of marketing strategies.

7.1. TRANSFERENCE OF KNOWLEDGE AND TECHNOLOGY IN THE SECTOR

Despite the scarce and scattered information about this specific activity in Portugal, the collection of shellfish is thought to play an important socio-economic role in the subsistence of small artisanal fishing communities and bivalves continue to be freely caught by people along the margins of the lagoons and coastal areas. Increasingly less full-time professionals are interested in joining the activity as the financial rewards have been decreasing year after year. Additionally, there are also less people available to work in fishing boats. Fisheries are seen by the younger generations as a non-attractive economic activity which involves a great deal of hard work for often very small financial compensation. Because of this perspective, only people with very little qualification actually join the fishing crews, often, exhausting the possibilities of finding any other type of job. This represents a real restraint to the development of the activity as these people are often unreliable, little motivated and rapidly leave if any other work opportunity shows, even if that implies an even lower income (Anonymous, 2010). Latest data showed that 647 (4.0%) full-time professionals had no qualification, 13 827 (86.2%) went to

elementary school, 1236 (7.7%) had middle education, 25 (0.2%) went to high school and only 313 (2.0%) were graduates (DGPA/INE, 2011). The existence of training programs, either provided by governmental or private entities, is crucial for the development of this sector. This would contribute to the requalification and training of farmers and producers, in order to obtain information on how to increase production efficiency and add value to the product, optimizing production, depuration, legislation, marketing, community organization, among other aspects.

The success of the sector involves the knowledge of the social, economic and cultural context of the target public, the knowledge of the marketing processes of the product and, during its implementation, the knowledge of the biological, zootechnical and environmental aspects, along with the food safety aspects and legislation and requires the governmental supervision inherent to products intended for human feeding. From this point of view, the involvement of a multidisciplinary teams with qualified professionals trained to deal with the diversity of tasks involved is urgently needed. Both universities and government institutes have been conducting work directed towards the aquaculture practices. Often, scientific research is directed to the ecosystem and natural resources and lacks commercial perspective. It is essential that research become compatible with the real needs of this sector, acting as a promotion factor. This requires the definition of research priorities, which should cover: (1) the implementation of chemical and microbiological monitoring for the classification and certification of both products and production areas, since at its present form, the process is obsolete and unrepresentative (Oliveira *et al.*, 2011); (2) the assessment of shelf lifetime for bivalve pre-cooked, cooled and frozen; (3) the modernization and improvement of the efficiency of the depuration process; (4) the study of alternatives for shellfish processing, (5) the evaluation of other bivalve species with potential to be used in aquaculture in order to achieve a higher diversification of the commercialized products enhancing competition with other countries. The dissemination and transfer of knowledge/technology between researchers, management agents and fishers would be a relevant factor in the production of shellfish. It is important to recognize that the process of technology implementation is slow, since it requires from the researchers a range of knowledge and skills, which often go beyond standard technical procedures. Thus, the integration of technical assistants (previously selected and trained) to support research

in the institute generating technology would facilitate the crossing of information as well as the achievement of fast and improved results.

7.2. THE ORGANIZATION OF THE SECTOR

Fishers have difficulty in being recognized as agents of promising activity. Because of the intrinsic characteristics of the activity and fishing communities, it is difficult to bring people together under organized entities that could centralize relevant steps of the production chain. Associations and cooperatives are common structures in the economy of other countries, namely Spain, created with the purpose of overcoming the derogatory professional status, the dispersion of efforts and resources and the weakening of the productive sector.

In 2010, there were 43 Portuguese organizations of fishers and fishfarmers, covering a total of 5564 professionals (DGPA/INE, 2011). Sessions of technical advisement for the constitution of associations or cooperatives, as well as lectures, seminars and workshops promoted by institutions working directly within the field would help to encourage the creation of these consortia. Even when fishers are organized in associations or networks, some difficulties are frequent, namely those related to: (1) financial and administrative bureaucracy (2) insurance coverage, (3) management of conflicts with other activities such as tourism and fishing and (4) the application for incentives for the development of the activity. Notwithstanding, the performance of these organizations has had positive effects in terms of competitiveness and productivity of the sector, security of persons and properties and guarantee of the quality of the product. However, the restructuration of these organizations would be desirable allowing an effective intervention in other areas, particularly in the marketing of the product and in the access to wider and more competitive markets.

7.3. SANITARY STATE OF HARVESTING AND PRODUCTION AREAS

The increase in population density, the industrialization, the need for better treatment of sewage disposals, the small river outlets or the diffuse land run-off are some of the ways by which bivalves become exposed to pollution, retarding growth and diminishing sanitary

quality (Mesquita *et al.*, 2011; Oliveira *et al.*, 2011). The classification of the production areas of bivalve molluscs according to the sanitary standards (in A, B and C) contributed to the traceability of the product leading to a faster evaluation of the treatment required prior to commercialization (Oliveira *et al.*, 2011). A joined effort must be done in the reduction of negative environmental impacts and in the improvement of harvesting and production areas. Restriction to bivalve harvesting is often applied when the area does not meet the required sanitary standards. Additionally, the increased rigor of the legislation has led to a decrease in availability of approved harvesting areas. Also, the current unfavorable economic conjecture demands the reduction of costs. Accordingly, the sampling sites used to assess the sanitary state of the production areas might be reduced resulting in the misjudgment of the real sanitary state of the production areas thus decreasing the number of areas acceptable for shellfish harvesting and production. Microbial analyses are essential to confirm the sanitary status of an area, but the assessment of the evolution of the sanitary status both in space and time, might give a more comprehensive view of the harvesting and production areas and contribute to a more efficient management of this activity. Predictive models are essential, since they can help to prevent economical losses associated with the deterioration of shellfish beds. Therefore, the accessibility, acquisition and legalization of the bivalve exploitation areas under extensive regime should be better documented and grounded creating conditions for the organized expansion of the sector along the coast taking into account the protection of natural resources and the development of the complementary economic activities.

Fishfarmers invested heavily and have uncontrollable losses caused by (1) environmental contamination, (2) human influence in wild areas, (3) environmental factors (such as predation and red tides) and (4) ecological disasters. Also, a certain loss of productivity is associated with the period of closure that occurs from 1 May to 15 June since January of 1998 (Regulatory Order Nº 11/80 of 7 May, paragraph 1, Article 15; Decree-Law Nº 261/89 of 17 August). The closed season occurs in all zones and subzones of the coastal ocean off the coast of mainland Portugal for some species of bivalve molluscs (Ruling Nº 1228/10 of 6 December). Although there might be a monetary compensation from the government during this period, it always involves financial losses for producers. The existence of studies defining the period of closure for each species is essential to minimize excessive economical losses.

7.4. ADDING VALUE TO THE FISHERY AND FISH FARMING PRODUCTS

Certification is a way of ensuring that the product complies with the requirements of high organoleptic quality. This would provide an added value to the product and to the mollusc marketing, providing a good return on the initial investment. Sanitary certification of a product from aquaculture raises the price, over the different levels of the market chain, because of its justified quality and safety for the consumer. The quality of certified products enhances consumer confidence which is indispensable for the socio-economic viability of this activity. Nevertheless, certification is often given after depuration of the bivalves. It is well established that depuration procedures need important improvements (Oliveira *et al.*, 2011) and usually result in a loss of weight and quality of the product. The use of bacteriophages during depuration as a way of rapidly achieving healthier bivalves would mitigate these losses with the advantage of excluding important pathogens as well as autochthonous bacteria (Oliveira *et al.*, 2011). Bacteriophage administrations during depuration would reduce the time needed for depuration before putting the product into the market consequently maximizing the commercial circuit. This methodology is already being used in other food products and suggested for bivalve molluscs (Oliveira *et al.*, 2011). Indeed, Intralytix is the only company in the world approved for using a bacteriophage-based preparation as a food additive. The use of depurated although less attractive products for canning industry, not only increases profitability of these products but it creates a possibility of expansion of this industrial area by creating a new market niche. Also, the development of gourmet products (nationally or regionally) and the attribution of quality labels for seafood products would help to valorize this sector of the economy. Given the current economic conjecture, it is also important to address the indirect returns which include the creation of new employment opportunities promoting economic and social stability of the coastal populations.

8. CONCLUSION

There is considerable potential for expansion of the bivalve exploitation sector and industry in Portugal. Despite the favorable natural conditions of the Portuguese territory for the effective development of bivalve exploitation, they have not been fully used for the

improvement of bivalve harvesting and production processes with evident economic losses. This progress requires joint action of governmental entities/companies/research centers to approach new strategies like (1) development of certified products and quality labels, (2) marketing strategies appealing to the benefits of seafood and its subsequent quality, (3) diversification of products, including other bivalve species and different presentations of the traditional products and (4) improving the knowledge and characterization (in a space and time scale) of favorable areas along the Portuguese coast without threatening environmental quality and animal welfare, towards a sustainable increase in production. There is a great need to involve and motivate fishers, producers and the general public improving the communication among stakeholders in order to promote the sector sustainability investing in innovation and in the quality of the product, making better use of all the fishing opportunities and taking advantage of the potentialities of aquaculture, with the purpose of adding value to the fishery and fish farming products (Anonymous, 2010).

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Chapter II

Microbial contamination and purification of bivalve shellfish: crucial aspects in monitoring and future perspectives

Microbial contamination and purification of bivalve shellfish: crucial aspects in monitoring and future perspectives - a mini-review

In: *Oliveira, J., Cunha, A., Castilho, F., Romalde, J.L. & Pereira, M.J. (2011). Microbial contamination and purification of bivalve shellfish: Crucial aspects in monitoring and future perspectives - A mini-review. *Food Control* 22: 805-816.

ABSTRACT

Shellfish are a nutritious food source whose consumption and commercial value has risen dramatically worldwide. Although bivalve's consumption can contribute to a healthy diet, some can cause foodborne illnesses. Microbial contamination is chronic and pervasive in harvesting areas and may be passed on to the consumers. Current food safety programs intend to protect consumers. Nevertheless, bivalve's microbial contamination is underestimated and undermanaged, which can pose a potential public health risk. We intend to provide an updated overview of the microbial assessment of bivalves and emerging alternatives or complementary perspectives for the elimination of microbial contamination. Further research is needed for the improvement of public health control and the enhancement of shellfish safety.

KEYWORDS: Bivalves; Microbial contamination; Shellfish safety.

*Published paper in Annexes.

1. INTRODUCTION

It is believed that less processed or natural foods are healthier. Nevertheless, for some products this may be an oversimplification and represents a greater risk to consumers. Bivalve shellfish fit this description (Murchie *et al.*, 2005). For dietetic, traditional or food availability reasons, consumption of bivalves has been rising dramatically worldwide (Johnson & Hayasaka, 1988; Fauconneau, 2002; Murchie *et al.*, 2005). On the other hand, microbial contamination is chronic and pervasive in growing and harvesting areas. By filter-feeding from the surrounding water, bivalves bioaccumulate natural occurring or anthropogenic contaminants, arising this contamination to the consumer (Lees, 2000). Contamination includes pathogenic species capable of producing diseases outbreaks (WHO, 2010). In general, HACCP procedures and product processing applied to food products are sufficient to protect consumers from the risk of diseases. However, shellfish, because of their unique nature have their own distinct aspects of harvesting, processing and handling. Furthermore, bivalves are minimally processed, and traditionally consumed raw or lightly cooked as a whole (visceras included) (Romalde *et al.*, 1994; Lees, 2000; Murchie *et al.*, 2005). Recently, there has been observed an increasing concern regarding food safety, particularly in molluscan shellfish products. Extensive efforts have been pursued to assure a safe supply of bivalves, but disease and death due to viruses and naturally occurring bacteria have been observed. This might be a result of underestimated and undermanaged microbial contamination.

This mini-review focuses on critical aspects related to shellfish safety for human consumption with the aim of serving as a general reference in future investigations. The drawbacks in depuration and relaying processes, encountering potential indicators for human enteric viruses as well as indigenous marine bacteria and the methodology applied to quantify conventional indicators are pointed out. Emerging perspectives regarding the elimination of microbial contamination and the enhancement of shellfish safety are also discussed providing guidelines for future work in monitoring the health of bivalves.

2. IMPORTANCE OF BIVALVES

Bivalves, as a food component, are characteristically tender, easily digested, additive-free and minimally processed. These characteristics make them a product that almost completely fulfils the demands of consumers (Murchie *et al.*, 2005). These animals also have high-quality animal protein content which is similar to that of milk and eggs making them a nutritive food and an important component in the human diet worldwide (Bernardino, 2000; Fauconneau, 2002; Murchie *et al.*, 2005; FAO, 2006; Sapkota *et al.*, 2008). This is particularly relevant in developing countries where aquatic products are often the only source of animal protein (Fauconneau, 2002).

The importance of bivalve shellfish as a food supply increases if we attend to the natural resource that shellfish growing areas may represent (Johnson & Hayasaka, 1988). Dense beds of bivalve shellfish (epifaunal or infaunal species) occur in inshore estuaries with high primary productivity and have been an important source of food since prehistory (Lees, 2000). However, the aquatic environment is becoming over-exploited and as a consequence of over-catching the depletion of stocks is leading to the reduction of natural shellfish beds and to the need of human intervention in its production (Pillay & Kutty, 2005). The outcome is the development of artificial bivalve shellfish production and exploitation by the food industry (Hernroth *et al.*, 2002). Aquaculture production has been exponentially increasing and becoming one of the fastest-growing food industries, especially in Asia (Defoirdt *et al.*, 2004; FAO, 2006; Sapkota *et al.*, 2008). Figure 1 shows aquaculture production both in quantity and in economic significance for fishes, molluscs, crustaceans and other aquatic animals in 2006 (FAO, 2009).

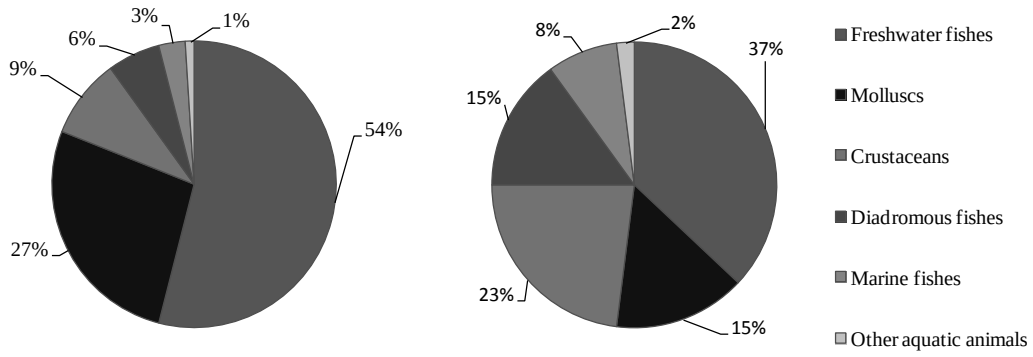

Figure 1 World aquaculture production in quantity (left) and respective economic significance (right) of major taxonomic families groups in 2006 (FAO, 2009).

Freshwater finfish represented half of global aquaculture production (54%) and molluscs were the second largest aquaculture product produced worldwide (24%) (FAO, 2009). The oysters culture, particularly *Crassostrea gigas*, dominates the global production of molluscs (Berthe, 2005; FAO, 2006). The Manila clam (*Ruditapes philippinarum*), the Yesso scallop (*Patinopecten yessoensis*), the blue mussel (*Mytilus edulis*) and the blood cockle (*Anadara granosa*) are also widely produced species (Berthe, 2005). Crustaceans come next in relevance, in terms of production, represented mostly by penaeid shrimps and grapsid crabs (FAO, 2006, 2009).

3. BIVALVES CONTAMINATION AND THEIR RISK AS VEHICLES OF DISEASE

Contamination of bivalve shellfish occurs mainly because they are suspension feeders that selectively filter small particles of phytoplankton, zooplankton, viruses, bacteria and inorganic matter from the surrounding water (Defossez & Hawkins, 1997; Burkhardt & Calci, 2000; Lees, 2000; Dunphy *et al.*, 2006).

For the majority of foods, proper refrigeration, storage, handling, cleaning and cooking procedures helps the consumer to control microbial risk and assure product safety. The hazards related to the contamination of bivalves by harmful microorganisms are due to their traditional cooking procedure which may not be enough to ensure consumer's safety. These circumstances make them an important vector of food-borne disease (Lees, 2000). The control of the disease risk associated with bivalves, thus, requires Hazard Analysis by Critical Control Point (HACCP) procedures together with water environment quality management of growing and harvesting areas and post-harvest product processing which might involve depuration and/or heat treatment where appropriate (WHO, 2010).

The true incidence of foodborne disease outbreaks is not known in Europe. Even thought there are routine surveillance systems worldwide that compiles the existing information on food-borne diseases, the collected information varies widely between diseases and between countries, not allowing for the numerical comparison of data on foodborne disease. Furthermore, a higher number of reported cases can be the result of a well performing surveillance system and not necessarily that people are more often sick from contaminated food. In addition, the reported number of cases for a country can include cases acquired domestically as well as acquired abroad after travel. No comparison between surveillance

systems in term of their efficiency can therefore be made in a realistic way, and subsequently, trying to compare various countries data according to their surveillance systems is not informative (Rocourt *et al.*, 2003).

In general, in countries who are members of the Organisation for Economic Co-operation and Development (OECD), meat (8,53%), poultry (4,14%), eggs and dairy products (14,62%) and seafood (6,63%) account for most of the foodborne diseases (Rocourt *et al.*, 2003). When compared with these highly-consumed food products, seafood represents a quite alarming vehicle for foodborne diseases Shellfish are identified as one of the mediums of seafood-borne diseases. In New York, from 1980 to 1994, 339 seafood-associated outbreaks were reported, resulting in 3959 illnesses, 76 hospitalizations, and 4 deaths. Seafood-associated outbreaks accounted for 19% of all reported foodborne outbreaks and 10% of foodborne illnesses. Shellfish, the most frequently implicated seafood item, accounted for 64% of seafood outbreaks. The etiologic agent was confirmed for 654 (36%) of 1802 foodborne outbreaks and 148 (44%) of 339 seafood-associated outbreaks. Of the seafood-associated outbreaks, 14 (9%) were attributed to bacteria, 69 (47%) to viruses, and 65 (44%) to chemical agents. Three of the 4 seafood-associated deaths were caused by *Clostridium botulinum;* the remaining death was caused by *Vibrio vulnificus* (Wallace *et al.*, 1999). From 1993 to 1997, a total of 2,751 outbreaks of food-borne disease involving 86,058 people were reported to the Centre for Disease Control (CDC), in Atlanta. The food vehicle was identified in only 1/3 of the outbreaks. Shellfish were often implicated in disease but did not, as opposed to some other foods, result in death. Since meat (66 outbreaks; 3205 cases) and poultry (52 outbreaks;1871 cases) are food products that are consumed in a much larger amount, when compared to seafood, the number of cases related to shellfish (47 outbreaks;1868 cases) is rather alarming (Olsen *et al.*, 2000). When compared to fish (140 outbreaks; 696 cases), molluscan shellfish caused double the number of cases even though being responsible for a much lower number of outbreaks (Olsen *et al.*, 2000; Huss *et al.*, 2004). In the majority of food outbreaks (67,8%) the disease agent was not identified. In 44,7% of the outbreaks caused by shellfish, the etiological agent was identified and viruses were the most frequent causative agent (23,4%) (Olsen *et al.*, 2000). Between 1995 and 1996, 1919 outbreaks of infectious intestinal disease, affecting more than 40 000 people in England and Wales were reported to the PHLS Communicable Disease Surveillance Centre (CDSC). The food vehicle was

identified for 301 outbreaks, 24 of which were reported to be due to shellfish, including 12 outbreaks attributed to eating oysters (Evans *et al.*, 1998).

The risk of disease or death due to contaminated shellfish consumption is inherent to all consumers but the risk increases in those that suffer from underlying health disorders and are exposed to the consumption of raw bivalves. Among the high-risk population are individuals with immunosuppressive disorders (cancer patients, AIDS), achlorhydria and epilepsy, patients with diabetes mellitus, liver and chronic kidney disease and steroid dependent patients (for treatment of asthma). Pregnancy, age and alcohol abuse are also factors that may enhance the development of seafood diseases (Ripabelli *et al.*, 1999; Butt *et al.*, 2004).

3.1. MICROBIAL CONTAMINATION AND HUMAN HEALTH

Foodborne disease is a public health problem which comprises a broad group of illnesses. Among them, gastroenteritis is the most frequent clinical syndrome which can be attributed to a wide range of microorganisms (Molnar *et al.*, 2006). Table 1 summarises some of the biological agents found in shellfish that can cause foodborne diseases. The risk of human intoxications is linked to the ingestion of bivalves contaminated with chemicals and biotoxins. On the other hand, the risk of human infections is related to the ingestion of bivalves contaminated with protozoan parasites, viruses and bacteria.

Chemical hazards (heavy metals, pesticides and drug-residues) are usually associated with aquaculture products or with bivalves caught from polluted waters but, in general, are uncommon in commercially harvested shellfish (Richards, 1988; Huss *et al.*, 2000).

Biotoxins, produced by dinoflagellates and diatoms (domoic acid), on the other hand are a serious health problem. These toxins, usually linked to the unpredictable growth of those microalgae (microalgae blooms), are heat resistant which means that even well cooked bivalves might still present a risk to consumer's safety. Accumulation of toxic marine algae in raw or light cooked shellfish has been associated to Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Neurotoxic Shellfish Poisoning (NSP), Amnesic Shellfish Poisoning (ASP) and Azaspiracid Poisoning (AZP) occurrences (Huss *et al.*, 2000; Hallegraeff *et al.*, 2003; FAO, 2004; Botana, 2008). The level at which PSP intoxications occur in humans varies considerably according to individual sensitivity and

Table 1 Some biological agents implicated in seafood-related illness (Adapted from Ripabelli *et al.*, 1999; Huss *et al.*, 2000; Hallegraeff *et al.*, 2003; Muniain-Mujika *et al.*, 2003; Butt *et al.*, 2004; Huss *et al.*, 2004; Brands *et al.*, 2005; Robertson, 2007; Botana, 2008).

Risk	Ethiology		Incubation period	Duration of pathology	Illness, symptoms and signs
Infection	**Bacteria**	*Salmonella* spp.	1- 3 days	4-7 days	Gastroenteritis and Enteric (typhoid) fever. Diarrhea, fever, vomiting, abdominal cramps.
		Shigella spp.	24-28 hours	4-7 days	Diarrhea, fever, abdominal cramps.
		Enterotoxigenic *E. coli*	1-3 days	3-7 days	Watery diarrhea, abdominal cramps, fever, vomiting.
		Campylobacter jejuni	2-5 days	2-10 days	Diarrhea, cramps, fever, vomiting.
		Staphylococcus aureus	1-6 hours	24-48 hours	Nausea, vomiting, abdominal cramps, fever, vomiting.
		Listeria monocytogenes	9-48 hours 2-6 weeks	Variable	Listeriosis, septicaemia, central nervous system infections (meningitis), gastroenteritis, endocarditis, arthritis, encephalitis, osteomyelitis, pulmonary infections. Fever, muscle aches, nausea, diarrhea, violent or bursting headache and convulsions.
		Vibrio vulnificus	1-7 days	2-8 days	Wound infections, septicaemia, gastroenteritis. Vomiting, diarrhea, abdominal pain.
		Vibrio parahaemolyticus	2-48 hours	2-5 days	Wound infections, septicaemia, gastroenteritis. Nausea, abdominal cramps, watery diarrhea, vomiting.
		Vibrio cholera	24-72 hours	3-7 days	Epidemic and non-epidemic gastroenteritis. Profuse watery diarrhea, vomiting and dehydration causing death with hours.

Table 1 Some biological agents implicated in seafood-related illness - continued.

Risk	Ethiology		Incubation period	Duration of pathology	Illness, symptoms and signs
Infection	**Viruses**	Noroviruses	24-48 hours	24-60 hours	Nausea, vomiting, watery large-volume diarrhea.
		Hepatitis A virus	15-50 days	2 weeks – 3 months	Diarrhea, dark urine, flu-like symptoms.
		Enteroviruses	10-70 hours	2-9 days	Nausea, vomiting, abdominal pain, malaise, headache, fever.
		Adenoviruses	10-70 hours	2-9 days	
	Protozoa Parasites	*Cryptosporidium* spp.	2-28 days	Days to weeks	Cramping, abdominal pain, watery diarrhea, fever, vomiting.
		Giardia lamblia	1-4 weeks	Weeks	Acute or chronic diarrhea, flatulence, bloating.
		Toxoplasma gondii	6-10 days	Months	Assymptomatic.
Intoxication	**Biotoxins**	Dinoflagellates and Diatoms	30 minutes – 3 hours	Hours to 2-3 days	Paralytic Shellfish Poisoning (PSP). Diarrhea, nausea leading to parethesias of mouth, lips, weakness, dysphasia, dysphonia, respiratory paralysis.
			30 minutes – 2 hours		Diarrhetic Shellfish Poisoning (DSP). Nausea, vomiting, diarrhea, abdominal pain, chills, headache, fever.
			Few minutes to hours		Neurotoxic Shellfish Poisoning (NSP). Tingling and numbness of lips, tongue, throat, dizziness, diarrhea, vomiting.
			24-48 hours		Amnesic Shellfish Poisoning (ASP). Vomiting, diarrhea, abdominal pain, neurological problems such as, confusion, memory loss, disorientation, seizure, coma.

fluctuation in the method of determination. For instance, an oral consumption of 300 µg PSP toxin per person was in some cases reported as fatal, whereas others noted the absence of toxic symptoms after an oral dose of 320 µg PSP toxin per person (FAO, 2004; Botana, 2008). Shellfish containing more than 2 µg Okadaic acid/g hepatopancreas are considered unfit for human consumption and capable of causing DSP (FAO, 2004). No mortality or chronic symptoms associated with NSP were reported and treatment is primarily supportive (FAO, 2004). For ASP the amounts of domoic acid consumed, ranged from 15 to 20 mg/person for an unaffected person to 295 mg/person for a case with severe neurological symptoms (FAO, 2004; Botana, 2008). Mild symptoms were showed after consuming 60 to 110 mg DA (0.9 to 2.0 mg domoic acid/kg body weight) and most serious cases were associated with consumption of 135 to 295 mg of domoic acid (1.9 to 4.2 mg domoic acid/kg body weight) (FAO, 2004; Botana, 2008). The *lowest-observed-effect-level* (*LOEL*) for AZP was 23 to 86 µg per person with a mean value of 51,7 µg/person (FAO, 2004; Botana, 2008).

The actual public health threat caused by parasites via shellfish consumption is uncertain, largely because there is minimal evidence of the transmission of infection (Robertson, 2007).

Microbial contamination is chronic and pervasive in harvesting areas. Furthermore, viruses and naturally occurring bacteria are the most often cited causative agents of disease and death related to shellfish consumption (Wittman & Flick, 1995; Huss *et al.*, 2000; Lees, 2000; Croci *et al.*, 2002).

Shellfish-derived illnesses can have natural causes due to contaminants that are available in the environment and consequently a part of the normal biota (Shumway & Rodrick, 2009), while others can be human-generated before or after shellfish harvesting. Pre-harvesting microbial contamination (occurring naturally or as a result of human activities) includes a wide variety of viruses and pathogenic bacteria (Huss *et al.*, 2000; Lees, 2000). Regardless of the higher abundance of indigenous marine viruses, only viruses derived from anthropogenic contamination are associated with illness in seafood consumers. Noroviruses, hepatitis A viruses, enteroviruses and adenoviruses are the viruses that are more often linked to shellfish contamination (Lees, 2000; Muniain-Mujika *et al.*, 2003; Le Guyader & Atmar, 2007). Shellfish may also be contaminated post-harvesting. Potential hazard due to sub-lethally injured microbiota that may recover and multiply during later

storage must be considered. Contaminant agents may also be introduced through cross-contamination, re-contamination or faulty handling and processing (Huss *et al.*, 2000; Shumway & Rodrick, 2009).

Viruses are frequently the cause of seafood-related infections, but hospitalisations and deaths are especially and generally related with bacteria (Butt *et al.*, 2004).

Among the indigenous microbiota of coastal environments, the family Vibrionaceae, particularly *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae*, is targeted as a causative agent of human disease due to the consumption of shellfish (Hood & Ness, 1982; Ripabelli *et al.*, 1999; Butt *et al.*, 2004; Normanno *et al.*, 2006). These natural pathogens remain viable and cultivable in water, even in the absence of organic matter (Croci *et al.*, 2002; Marino *et al.*, 2005; Pruzzo *et al.*, 2005).

Several reports of human disease caused by *Listeria* spp., namely listeriosis, were related to seafood consumption but inconsistent results were observed (probably as a consequence of distinct coast contamination or different efficiencies in the detection and quantification methods). Furthermore, the contamination source (marine environment and processing/ handling) and the seasonal fluctuations of the occurrence of these bacteria were not investigated effectively (Monfort *et al.*, 1998; Butt *et al.*, 2004; Rodas-Suárez *et al.*, 2006). It is worth highlighting that there is growing evidence of the emergence of multiresistant *Listeria monocytogenes* strains, due to the constant use of antimicrobial agents, thus representing a potential threat to human health (Rodas-Suárez *et al.*, 2006).

The presence of *Salmonella* spp. in seafood and water may cause salmonellosis, characterized by enteric (or typhoid) fever along with gastroenteritis, abdominal cramps and diarrhea (Brands *et al.*, 2005). *Salmonella enterica* serovar *Enteritidis* and serovar *Typhimurium* are the most common salmonella that cause infection and death (Butt *et al.*, 2004). Enterotoxigenic *Escherichia coli*, *Campylobacter jejuni* and *Staphylococcus aureus* are also among bivalve bacterial contaminants and agents responsible for human disease (Butt *et al.*, 2004; Brands *et al.*, 2005).

3.2. MICROBIAL CONTAMINATION SOURCES

The microbiological safety of bivalves as well as the suitability of coastal areas for growing and harvesting shellfish is directly related to the quality of the water in which they

grow (Son & Fleet, 1980). However, water quality does not necessarily reflect the sanitary quality of shellfish harvested (Burkhardt *et al.*, 1992). The increase in population density has increased the vulnerability of shellfish growing areas through shellfish exposure to human and industrial contaminants (Lees, 2000; Brands *et al.*, 2005). Sources of human and animal fecal pollution include pet and wildlife waste, rainfall events, and river flows. Uncontrolled sewage disposal or performed without previous appropriated treatment, small river outlets or diffuse land runoff of contaminants derived from agricultural activities and septic tank leakages may also produce sporadic contamination (Hernroth *et al.*, 2002). Shellfish growing areas are usually close to wastewater discharges or in polluted estuarine systems and bivalve contamination is usually linked to the accumulation of human and animal pathogens of fecal origin. Nevertheless, in the process of filter-feeding, bivalve shellfish are likely to accumulate a diversity of microbiological contaminants (Burkhardt & Calci, 2000; Croci *et al.*, 2002). Considering that fecal associated pathogens available in the marine environment accumulate in bivalves by filter feeding, thus sewage contaminants may be recycled into the human community (Hernroth *et al.*, 2002). This gains particular importance due to the fact that bivalves may have been exposed to persistent antibiotic residues and to multi-resistant pathogens as a result of an increased use of antibiotics by humans, in aquaculture and livestock. These multi-resistant pathogens may exist in the environment and may re-enter the food chain (Hektoen *et al.*, 1995; Lees, 2000; Rodas-Suárez *et al.*, 2006; Sapkota *et al.*, 2008). Furthermore, a nonculturable but viable and latent bacteria species of sanitary importance may be present in water besides the existence of various processes that control the levels of microorganisms in coastal marine environments (Troussellier *et al.*, 1998).

Allochthonous microorganism's number may be reduced in the natural environment because of physiological, hydrodynamic and biotic factors. Some of these are: pH, temperature, salinity, oxygen concentration, amount of organic matter, sunlight, water dispersion, re-suspension, sedimentation, competition of autochthonous bacterial community for nutrients and, finally, microbial predation by planktonic organisms (Hood & Ness, 1982; Troussellier *et al.*, 1998; Ho & Tam, 2000). The same factors cannot be applied straightforward to microorganisms naturally present in water that also constitute a health problem (Croci *et al.*, 2002; Pruzzo *et al.*, 2005).

3.3. FACTORS WITH INFLUENCE ON MICROBIAL CONTAMINATION OF BIVALVES

Environmental conditions influence the occurrence of microorganisms in seawater and, consequently, their contact with shellfish. Burkhardt and his colleagues showed that temperatures outside the range of 13 to 22°C and salinities greater than 25 ppt reduce the survival of *Vibrio vulnificus* in seawater (Burkhardt *et al.*, 1992). Annual variation of water temperature and salinity influence shellfish's physiological state and capacity of siphoning and therefore affects the bivalve's ability to selectively accumulate microbial species. Kaspar and Tamplin described that the greatest accumulation of microorganisms in hard-shelled clams occurred during certain periods in the spring, at temperatures ranging from 11.5 to 21.5°C (Kaspar & Tamplin, 1993).

Furthermore, bivalve's inter- and intra-specie variations determine the amount of water filtered, which is between twenty and one hundred liters *of water* a day, independently of the environmental conditions (Richards, 1988; Robertson, 2007). This means that, bivalve molluscs feeding physiology determines the accumulation of pathogenic microorganisms filtered from the overlying water (Burkhardt & Calci, 2000; Ho & Tam, 2000). These phenomena may partially explain seasonal and geographical differences in microbial content of bivalves (Hernroth *et al.*, 2002). The availability of edible shellfish depends on the fluctuation of microorganism (type and quantity) in the marine environment as contamination results from ingestion of accessible contaminants. The ability of accumulated microorganisms to persist and multiply in bivalve tissues, despite the natural protection of the shellfish by the bactericidal activity of the haemolymph, also influences the existence of unhealthy shellfish (Johnson & Hayasaka, 1988; Power & Collins, 1990; Pruzzo *et al.*, 2005).

4. ENSURING SAFE HUMAN CONSUMPTION

4.1. CONTROLLING HARVESTING AREAS

A few years ago, investment in sewage treatment processes still had many barriers to overcome. The geographical location of the shellfish industry was used as an argumentative factor to justify the difficult and expensive task in achieving and maintain

high standards of water quality. Investing in adequate sewage treatment systems was considered disproportionate in terms of the value of the shellfish industry (Lees, 2000). Environmental concerns have contributed in recent years, to the increased investment in sewage infrastructure. However, important improvements are still needed, namely appropriate discharge locations for treated water, adequate arrangements for storm water storage and treatment, tertiary treatment of effluents and adequate evaluation methodologies of the effluent microbial quality (Lees, 2000). The location of pollution inputs must be previously well identified in order to assure that quality-monitoring programs take them into consideration. This may result in the expansion of sewage infrastructures even to sparsely populated areas or other areas which represent a low sewage input (Lees, 2000). Risk management strategies for shellfish harvesting areas must be improved in other to prevent shellfish contamination (Shumway & Rodrick, 2009).

4.2 LEGISLATION FOR SAFEGUARDING CONSUMERS

Adequate safeguards can be useful in minimizing the probability of shellfish microbial contamination, from harvesting to consumption, and in the protection of public health. The European Directive 2006/113/CE (Anonymous, 2006) and the European Directive 2004/41/CE (Anonymous, 2004d), the US interstate agreement set out by the Food and Drug Administration (Anonymous, 1993) or the UK Advisory Committee on Microbiological Safety of Food (Anonymous, 1998) are guidelines, based on the levels of microbiological indicators for both shellfish and overlying waters. The legislation employs a classification to the seafood harvesting areas according to bacterial indicators of sanitary quality (*Escherichia. coli*), quantified through a 5-tube 3-dilution most probable number (MPN) test. This classification determines whether shellfish can be sent for direct consumption or must be treated previously to commercialization (Lees, 2000). Table 2 summarises the European standards for bivalve shellfish beads. All shellfish sent for direct human consumption without any further processing must comply with a standard of less than 230 *E. coli* in 100 g of shellfish meat in more than 90% of samples. Shellfish harvesting from polluted (category B and C) areas is allowed when shellfish undergo previous treatment, before being commercialized. Bivalve molluscs harvested from growing areas exceeding Category A standards can be placed on the market for human

consumption following controlled self-purification in tanks of clean seawater (commercial depuration), prolonged relaying in clean seawater or commercial heat treatment or processing by any other acceptable method (Jones *et al.*, 1991; Lees, 2000; Murchie *et al.*, 2005). Shellfish from category C areas may, if necessary, be depurated before commercialization. However, some processes may not be effective at high levels of contamination, so another category is defined as D. Shellfish from those harvesting areas cannot be treated by any of the procedures previously mentioned.

The final product is sealed, labelled for traceability and commercialized giving distributors and consumers the confidence of a safe certified product (Jones *et al.*, 1991; Lees, 2000; Shumway & Rodrick, 2009).

Table 2 European classification of bivalves growing areas according of *Escherichia coli* (Lees, 2000).

Category	MPN of *Escherichia coli* per 100g of seafood	Treatment required
A	≤230	Direct human consumption.
B	]230; 4 600]	Depuration or relaying, to meet category A.
C	]4 600; 46 000]	Protracted relaying to meet category A. Relaying to meet category B and depuration.
D	>46 000	Harvesting prohibited.

The U.S. Food and Drug Administration control procedures similarly rely on microorganism indicators for monitoring harvest waters in order to determine approved and restricted harvest areas and the treatment requirements prior to being released for human consumption (Lees, 2000). Category A defines the cleanest growing areas from which shellfish can be harvested and these areas are classified as “approved”. Bivalve growing areas that do not comply with satisfying criteria, or without classification due to the lack of sanitary surveys, cannot be harvested for human consumption and are defined as “restricted”. Harvest restriction can also be employed for short periods of time as a result of predictable or sporadic pollution. Such areas are classified as “conditionally

approved" or "conditionally restricted" (Lees, 2000). The frequency of sample collection is dependent on the degree of contamination of the harvesting areas (Richards, 1988).

In many countries, these standard guidelines become very important for the regulation of shellfish harvesting and routine monitoring of overlying waters (Jones *et al.*, 1991). However, when authorized shell fishing harvesting areas decrease, non-ethical activities such as illegal harvesting from polluted and restricted areas, wet storage of harvested shellfish in polluted waters, and other violations of legislation become problematic (Jones *et al.*, 1991).

Other important aspects, other than the classification of growing areas, must be considered in order to reduce shellfish contamination. To achieve consumer protection and to minimize the inherent risks of shellfish consumption, legislation also sets requirements for sample collection, wet storage, bivalve self-purification by depuration and/or relaying (tank construction and operation, packaging, labelling), shellfish processing, laboratory analytical methodologies and product distribution. Regulations on food hygiene (Regulation Nº 852/2004/EC) and on living bivalve molluscs (Regulation Nº 853/2004/EC-Annex III Section VII) are well understood. Other regulations impose microbiological criteria for foodstuffs that set acceptable microbiological limits for all foods including live bivalve shellfish (Regulation Nº 2073/2005) (Anonymous, 2004a, b, 2005). Regulation Nº 854/204/EC establishes specific attributes on the organisation of official controls on products of animal origin intended for human consumption (Anonymous, 2004c). At all stages, starting from the moment that the shellfish is collected until its consumption, good handling practices by applying Good Manufacturing Practice (GMP), Good Hygiene Practice (GHP) and a well designed HACCP programme are needed to prevent contamination and ensure a safe product (Huss *et al.*, 2000; Lees, 2000; Marino *et al.*, 2005; Shumway & Rodrick, 2009). Despite all regulations and guidelines, Sagoo and his colleagues showed that, in the UK during 2003, molluscan shellfish from retail and production premises found that 4% of 682 batches were unsatisfactory due to the presence of high levels of *Escherichia coli* (3.3%; 102 to 106 cfu g^{-1}), *Vibrio parahaemolyticus* (0.4%; 102 to 106 cfu g^{-1}), and *Staphylococcus aureus* (0.3%; > 103 cfu g^{-1}) (Sagoo *et al.*, 2007).

5. PURIFICATION METHODS

Sanitary regulations rely on bacterial indicators of sewage contamination to classify shellfish harvesting waters and to estimate the efficiency of purification methods (Murchie *et al.*, 2005). These purification procedures, used to reduce anthropogenic or natural microbial contamination of bivalve molluscs, have been used since the 1920s and are now extensively used worldwide (Lees, 2000). Unhealthy harvested bivalves purge contaminants when transferred into clean natural shellfish beds (relaying) or into tanks (depuration) (Richards, 1988; Shumway & Rodrick, 2009). Depuration consists of a flow-through or recirculation system of chemically (chlorine, ozone, iodophores, and activated oxygen) or physically (UV irradiation) disinfected water to allow purification under controlled conditions (Son & Fleet, 1980; Richards, 1988; Lees, 2000). This process usually occurs in 2 days (Lees, 2000). Relaying consists of transferring contaminated harvested bivalves to cleaner areas allowing self-purification in the natural environment for longer periods, at least two months for category C shellfish, according to EU standards (Richards, 1988; Lees, 2000). Purification processes are based on the assumption that if by filtering polluted water shellfish can become contaminated, they may also purge the contaminants by filtering clean water. Thus, microbial depuration decreases the risk for potential infections due to shellfish consumption. In fact, most consumers prefer to buy depurated products, not only because they are safer in terms of contamination, but also because they are less gritty and more palatable (Richards, 1988).

5.1. DEPURATION – PRACTICAL CONSIDERATIONS

Depuration efficiency is primarily related to bivalve's size, siphoning activity, and physiological conditions (Richards, 1988; Jones *et al.*, 1991).

The type and quantity of initial contamination also accounts for depuration efficiency as more contaminated bivalves require longer depuration times and different microorganisms respond differently to the purification process. Likely, seeded (laboratory-induced) and natural-contaminated bivalves present dissimilar kinetics of contaminant elimination (Son & Fleet, 1980). Artificially contaminated molluscs depurate more rapidly than environmentally contaminated ones (Richards, 1988; Jones *et al.*, 1991; Croci *et al.*, 2002).

Different rates of elimination also occur when bivalves are contaminated with individual or several bacteria (Son & Fleet, 1980).

Temperature and salinity are two important parameters to consider in the purification process according to the type of shellfish. Variations in environmental requirements among bivalves may reflect shellfish adaptation to *in situ* conditions. Animal stress induced by differences in water temperature, from that of the *in situ* shellfish growing areas to the process water, also influence purification time and efficiency. Lowering the temperature may help in keeping bivalves alive longer and maintain lower bacterial concentrations, however, this would also extend the period of time required for effective depuration. Shellfish conditioning, that allows shellfish to acclimate to the temperature and salinity of the water, seems necessary to ensure maximum depuration (Johnson & Hayasaka, 1988; Richards, 1988). Specific studies are required to determine optimal conditions for shellfish microbial depuration accordingly to geographical characteristics (Johnson & Hayasaka, 1988). Differences in experimental design, such as commercial or laboratory-scale depuration systems must also be considered, as the time needed for bivalve purification differ (Jones *et al.*, 1991). Susceptibility to temperature fluctuations is less likely in thermostatically controlled systems. Also, water volume and shellfish loading rates will affect the pH and the dissolved oxygen levels in the system. The number of bivalve layers in depuration recipients can promote increases in the microbial load as result of recontamination, obstruction of water flow and restrictions of shell opening (Richards, 1988). Depuration has a great potential as a means of purging shellfish, at least partially, of microbiological contaminants. Nevertheless, more detailed studies are needed to determine the effect of physiologically parameters, such as food availability, temperature, salinity, dissolved oxygen and shellfish state. This would allow the development of an improved depuration method (Jones *et al.*, 1991).

5.2. RELAYING - DRAWBACKS

In contrast to depuration, where bivalves can only be held for a short period of time (maximum of 48 hours), in the relaying method, molluscs can be kept for longer periods (at least two months) (Richards, 1988; Lees, 2000). In fact, in controlled purification, extended periods will reduce palatability and quality of bivalves and might even, cause

bivalve mortality due to the unavailability of food. This will obviously result in a negative economic impact, due to delayed marketing and commercialization (Jones *et al.*, 1991).
Drawbacks of relaying include: lack or availability of acceptable sanitary shellfish growing waters, early harvesting from fishermen and economical considerations namely regarding ownership rights (Richards, 1988; Lees, 2000). In addition, bivalves are more susceptible to environmental disturbances that cannot be controlled such as temperature fluctuations, water movements (tides and storms) and weather (Son & Fleet, 1980; Richards, 1988; Lees, 2000). Smothering and clogging by sediments, physiological stress, shell damage and predation are very likely to occur during the relaying process (Richards, 1988). Furthermore, water quality of relaying areas is difficult to assure. The possibility of recontamination by seasonal variations of naturally occurring bacteria populations or transient pollution (due to heavy rains and associated land runoff), may contaminate acceptable relay areas, leading to an ineffective microbial reduction (Richards, 1988; Ho & Tam, 2000; Lees, 2000; Croci *et al.*, 2002). Assessing the efficiency of the relaying process is also difficult because the indicator microorganism's levels may fluctuate erratically during the exposure period (Richards, 1988; Ho & Tam, 2000).
In summary, eating raw or lightly steamed shellfish harvested from contaminated areas, but purified in acceptable marine waters or in artificial tanks, can still cause infection and disease in a significant percentage of the exposed population (Richards, 1988; Lees, 2000).

5.3. MICROORGANISMS INDICATORS – IMPORTANT CONSIDERATIONS

Conventional depuration can be a viable alternative for molluscs that have been exposed to polluted waters improving their quality as a food resource, especially for those that are sold alive for raw consumption - it reduces the bacteria levels present in mollusc meat without heat processing (Johnson & Hayasaka, 1988; Jones *et al.*, 1991; Lees, 2000).
However, the efficiency of these purification practices is questionable since it is based on bacterial indicator standards to ensure shellfish safety. The use of such indicators was made necessary by the difficulty in detecting many human pathogenic bacteria and viruses. Additionally, they avoid the need to screen for individual fecal pathogens (Scott *et al.*, 2002). Nevertheless, there is a well known lack of correlation between the presence of bacterial indicators and viral pathogens (which are tightly attached to the internal tissues)

in both shellfish and harvesting waters. Dissimilar elimination rates of indicator bacteria compared to viruses and indigenous marine bacteria are also well documented (Son & Fleet, 1980; Romalde *et al.*, 2002; Marino *et al.*, 2005; Murchie *et al.*, 2005). Hence, more representative and accurate indicators are sought in order to improve the microbial control of shellfish (Formiga-Cruz *et al.*, 2003).

The occurrence of few pathogenic bacteria in shellfish does not generally represent a high risk to public health because threshold levels necessary to cause illness far exceed those present. In contrast, viruses are infectious even in very low numbers, which makes total virus depuration essential to ensure public safety (Richards, 1988; Lees, 2000). Disease outbreaks associated with the consumption of shellfish compliant with the *E. coli* standard (less than 230 *E. coli* per 100 g), particularly in relation to viral infections, continues to be reported (Doré *et al.*, 2000; Lees, 2000). It seems that viruses survive longer both in the marine environment and in the digestive tracts of bivalves compared to *E. coli* (Hernroth *et al.*, 2002). Furthermore, there are studies reporting the detection of viruses in shellfish harvested from areas considered unpolluted, and meeting the current bacteriological standards (Romalde *et al.*, 2002; Muniain-Mujika *et al.*, 2003). Viral pathogens include culturable and nonculturable viruses whose detection methods are complex, laborious, time-consuming and expensive. Consequently, their use in routine monitoring is limited, hindering their establishment as regulatory standards methods (Lees, 2000; Hernroth *et al.*, 2002; Murchie *et al.*, 2005).

5.3.1. INDICATOR MICROORGANISMS – ALTERNATIVES

The analysis of fecal coliforms and *E. coli* has limited predictive value for viral pathogens such as, noroviruses (NV), hepatitis A viruses (HAV), enteroviruses (EV) and adenoviruses (ADV), and alternative indicators microorganisms have been proposed (Muniain-Mujika *et al.*, 2003). Traditional depuration does not significantly reduce the levels of Male-specific RNA (F-RNA) bacteriophages, somatic coliphages, bacteriophages infecting *Bacteroides fragilis*, or the occurrence of human pathogenic viruses, although its efficiency in reducing *E. coli* levels was confirmed (Formiga-Cruz *et al.*, 2003). Based on these findings, the phages above mentioned *have been suggested as putative indicators of viral contamination (Hernroth et al., 2002).* F-RNA phages, frequently found in sewage and fecal contaminated waters, are a group of single-stranded RNA viruses that belong to

the family Leviviridae and their physical and genomic properties are similar to the NV and HAV (Doré *et al.*, 2000; Doré *et al.*, 2003). F-RNA bacteriophages are probably more representative of the pathogenic viral kinetics in shellfish than *E. coli*, either because they are more resistant to environmental stress (U.V. irradiation), or because they have longer retention time in shellfish (due to the differences in the way they are accumulated and eliminated) or even a combination of the two (Doré *et al.*, 2003). Virus depuration is slower than indicator bacteria clearance, requiring more than 48 hours and still does not always meet acceptable criteria (Richards, 1988; Lees, 2000). In fact, recent studies suggested a 5-day depuration treatment to ensure elimination of viruses in mussels (Formiga-Cruz *et al.*, 2003). Hence, the slower elimination kinetics of F-RNA bacteriophages in relation to *E. coli*, during depuration, appears to be representative of the kinetics of elimination of human enteric viruses (Hernroth *et al.*, 2002). These properties associated to the simplicity of enumeration, make F-RNA phage an attractive indicator organism for viral contamination in the marine environment (Hernroth *et al.*, 2002; Doré *et al.*, 2003). However, some authors have presented some reservations in terms of the fact that monitoring through this indicator will increase shellfish safety (Hernroth *et al.*, 2002; Torrado *et al.*, 2002; Vilariño *et al.*, 2006). Indeed, F-RNA phages have demonstrated a significant relationship to the presence of human viruses in shellfish, although showing very weak predictive capability for EV, HAV and ADV and a stronger predictive capability for NV (Formiga-Cruz *et al.*, 2003). On the other hand, the absence of F-RNA bacteriophages appears to be a reliable indicator that enteric viruses, such as NV, are likely absent (Doré *et al.*, 2000). Similarly to *E. coli*, F-RNA bacteriophages are not human specific, and a contamination with this phage may be associated to animal feces originated by land runoff and may not imply health risk due to NV (Doré *et al.*, 2000). Oligonucleotide probe hybridization methods for genotyping F-RNA bacteriophages would provide the possibility to differentiate animal-associated from human-associated bacteriophage groups (Doré *et al.*, 2000). Somatic coliphages, viruses that infect *E. coli* bacteria, are constantly present in treated or non-treated sewage, they are non-pathogenic to humans, and are more similar to enteric viruses with respect to physical characteristics, environmental resistance to inactivation in the marine environment and resistance to treatment processes than are indicator bacteria (Cole *et al.*, 2003). However, coliphages are able to increase their initial effluent discharge number in marine environment and in

shellfish. Furthermore, they are not a specific index for pollution with human enteric viruses, as they are found in both human and other animals (Legnani *et al.*, 1998). Male-specific (F+) coliphage (group II and III) has been pointed out as providing an additional advantage in distinguishing animal and human fecal pollution (Scott *et al.*, 2002; Cole *et al.*, 2003). The *Bacteroides* spp. is present in high numbers in both the human and animal gut and is a major component of human feces (Scott *et al.*, 2002). Several studies have reported that the probability of detecting viruses increases when phages of *B. fragilis* are found, particularly, *B. fragilis* RYC2056 (Muniain-Mujika *et al.*, 2003). The detection of *B. fragilis* phage has the advantage of being highly specific. Additionally, these phages do not replicate in the environment (Scott *et al.*, 2002). This could be a suitable group of bacteriophages to be used as an indicator of the presence of viruses in shellfish (Muniain-Mujika *et al.*, 2003).

Some authors propose human ADV as a molecular index of viral contamination in shellfish (Pina *et al.*, 1998; Hernroth *et al.*, 2002; Muniain-Mujika *et al.*, 2003). In fact, this virus was usually detected when EV and HAV were also found (Hernroth *et al.*, 2002). Technical simplicity related to simpler detection methodologies of DNA viruses compared to those of RNA viruses and more sensitive and specific molecular techniques, are the advantages of using human ADV as a molecular indicator of human-specific viral fecal pollution (Lees, 2000; Hernroth *et al.*, 2002; Muniain-Mujika *et al.*, 2003). However, epidemiological studies for EV and ADV are difficult to perform because those infected by the viruses can act as carriers without showing any symptoms. As a result, the disease may only become apparent after the infection of another individual, probably far away from the original source (Hernroth *et al.*, 2002; Muniain-Mujika *et al.*, 2003). However, detection of human ADV by PCR has been proposed as a molecular parameter for monitoring the presence of human viruses in the environment, more studies are required to define the relationship between the level of viral contamination in shellfish and their potential pathogenic effect after consumption (Muniain-Mujika *et al.*, 2003). Furthermore, ADV are present in much higher numbers than HAV or NV and therefore their value as indicators are limited (Torrado *et al.*, 2002).

It is important to notice that environmental conditions play an important role in the accessibility, accumulation and elimination of both viral contaminants and potential indicator organisms from bivalves (Hernroth *et al.*, 2002).

Temperature and UV irradiation are some of the factors affecting the viability and stability of viral particles in seawater and virus removal during depuration (Lees, 2000; Formiga-Cruz *et al.*, 2003). Somatic coliphages have been indicated as ensuring a better marine water quality monitoring than F-RNA phages and fecal coliforms because the formers are less susceptible to longer solar wavelengths, which are predominant in the marine environment (Sinton *et al.*, 1999). It was found that the probability of a positive detection of any of the pathogenic virus decreases as the temperature of shellfish growing waters increases (Muniain-Mujika *et al.*, 2003). The levels of the potential indicators also change with temperature. The distribution of F-RNA bacteriophages has been shown to be seasonal, with higher levels during the winter; this trend was also observed in the identification of typified NV, but not for the detection of ADV, EV, or HAV (Hernroth *et al.*, 2002; Doré *et al.*, 2003; Formiga-Cruz *et al.*, 2003). In fact, NV gastroenteritis has been considered a "winter vomiting disease" (Doré *et al.*, 2000; Hernroth *et al.*, 2002). Phages infecting *B. fragilis,* in contrast to ADV, decrease in number with temperature (Hernroth *et al.*, 2002). The selection of an indicator microorganism is further complicated when focusing on the potential pathogenicity of some indigenous marine bacteria (Murchie *et al.*, 2005). Autochthonous bacteria are not implicitly associated with the presence of fecal contamination. Thus classical indicators of fecal contamination do not predict their presence in shellfish or water (Hood & Ness, 1982). Furthermore, one of the basic criteria for a good indicator organism is that the indicator must survive as long as the pathogen, but *E. coli* does not survive in estuarine water as well as *V. cholera* (Hood & Ness, 1982). Several authors have confirmed the lack of correlation between traditional indicators and the presence of *Vibrio* spp. (Hood & Ness, 1982; Ripabelli *et al.*, 1999; Marino *et al.*, 2005; Normanno *et al.*, 2006). Seasonal variations in the indigenous bacteria populations make it extremely difficult to select safe waters for mollusc harvesting (Croci *et al.*, 2002). Fecal indicators provide an inadequate index of microbiological safety for naturally occurring vibrios and underestimate the efficiency of the depuration process. Like enteric viruses, *Vibrio* spp. has a different response to the depuration process from that of *E. coli.* It is possible to obtain edible shellfish from anthropogenically-contaminated shellfish, but the same measure cannot be used with shellfish contaminated by naturally occurring bacteria. Similarly, it is expected that the elimination of microorganisms derived from fecal contamination and those included in shellfish natural microflora would be different (Jones

et al., 1991; Croci *et al.*, 2002). In fact, indigenous marine bacteria do not depurate well and may even multiply in depurating shellfish tanks and pumping systems (Richards, 1988). Therefore, a more appropriate indicator must be developed to reduce seafood illness risk derived from *Vibrio* spp.. Enterococci have been proposed as a more appropriate indicator of the risk from vibrios than *E. coli* (Marino *et al.*, 2005). It is also important to be aware of the fact that none of the current regulations include specific tests for indigenous marine bacteria (Murchie *et al.*, 2005). Thus, the need to improve shellfish-borne disease control strategies must also focus its attention on *Vibrio* spp. (Ripabelli *et al.*, 1999).

5.4. METHODOLOGIES FOR MONITORING BIVALVE'S SAFETY – CRITICAL POINTS

Present legislation verifies seafood safety according to bacterial indicators of sanitary quality measured through a 5-tube 3-dilution most probable number (MPN) test (Lees, 2000). Besides the wide acceptance, it is recognized that this test presents interpretive, technical and microbial problems leading to the underestimation of both bacteria indicators and contamination-level and is therefore of limited reliability. The MNP is a statistical estimate of the mean number of bacteria in the sample, thus the result is a semi-quantitative enumeration of bacteria indicators. The precision of the bacteria estimation is low and is dependent on the number of tubes used in the laboratory analysis (Rompré *et al.*, 2002). For this reason, this indirect enumeration procedure is intrinsically less accurate than the direct methodologies, unless the population densities are low. MPN method is time consuming due to the duration of the incubation; it is also tedious and laborious (Hackney *et al.*, 1979; Rompré *et al.*, 2002). The accuracy of this method is further significantly reduced by the interference of antagonistic bacteria, a certain degree of heterogeneity of the coliform group, the inhibitory nature of the media and weak detection level of slow-growing, stressed or viable or active but nonculturable microorganisms (Rompré *et al.*, 2002). Nonlethal injury may be caused either by temperature, pH, water activity, irradiation, sanitizers, starvation or by a combination of these factors (Hackney *et al.*, 1979). Specially developed media with the appropriated composition may help to recover these stressed or injured cells. Some advantages of this method are: its simplicity, low cost and no need of sophisticated laboratory and equipment. Improvements to the MPN test

have been developed over the years. Biochemical tests, based on metabolic reactions, can be used for culturable bacteria identification and enumeration. However, they are not totally specific, and supplementary confirmation tests are necessary. Microbial enzyme profiles can be used to detect indicator bacteria as a complement or alternative to the classical method (Rompré *et al.*, 2002). Nevertheless, innovative methods of bacterial detection and quantification are needed. Molecular methods have appealing characteristics such as sensitivity, specificity, the short time needed to produce results and the fact that they do not require complex culture or additional confirmation procedures, thus allowing for the detection of both culturable and nonculturable bacteria (Pina *et al.*, 1998; Hernroth *et al.*, 2002; Rompré *et al.*, 2002). Additionally, they allow for the detection of more than one microorganism or molecular marker with a single assay (multiplex-PCR) (Scott *et al.*, 2002). Polymerase chain reaction (PCR) or reverse transcriptase-PCR (to detect RNA viral genomes, such as those from viruses) is the most frequently applied nucleic-acid-based method (Pina *et al.*, 1998; Rompré *et al.*, 2002; Le Guyader & Atmar, 2007; Shumway & Rodrick, 2009). Despite the success of PCR and reverse transcriptase (RT)-PCR in detecting minimal starting quantities of nucleic acid (as little as one cell equivalent), the drawbacks of PCR-based assays included low amplification due to the presence of inhibitor substances, and the absence of information about the physiological activity of the bacteria or viruses being studied, because nucleic acids are extracted from viable, dead, culturable or nonculturable microorganisms (Rompré *et al.*, 2002). Some attention must also be paid to results given by methods based on PCR amplification of viruses because they might overestimate the risk for transmission of viable viruses. In addition, molecular approaches can only be performed with highly skilled staff in specialized laboratories providing high-technology services (Hernroth *et al.*, 2002; Rompré *et al.*, 2002; Le Guyader & Atmar, 2007). Real-time PCR overcome the lack of quantification in molecular methods by measuring PCR product accumulation through a dual-labeled fluorogenic probe (i.e., TaqMan Probe). As this method does not require post-PCR sample handling, it also avoids potential contaminations of the PCR product. Real-time quantitative PCR is extremely accurate, reproducible and less labour-intensive than other quantitative PCR methods that also had been designed, and can be applied to both virus and bacteria (Heid *et al.*, 1996).

6. EMERGING PERSPECTIVES

The emergence of *Vibrio* spp. as a human pathogen is of particular concern for shellfish producers. In addition, bivalves contaminated with these bacteria are difficult to recognize since they are not affected in appearance, palatability or smell. Several elimination methods have been proposed: UV depuration, gamma radiation, heat, cold temperatures, tabasco sauce and other horseradish-based sauces. Regardless of their success and limitations, these processes do not represent an alternative for raw seafood (Shehane & Sizemore, 2002).

Bacteriocins (plasmid-derived proteins used as microbial defense systems) have been studied as a method for the removal of *Vibrio* spp. from seafood (Riley & Wertz, 2002; Shehane & Sizemore, 2002). Three bacteriocin-producing strains (IW1, BC1 e BC2), belonging to the group IV bacteriocins of lactic acid bacteria, have been found to exhibit a varied inhibitory spectrum and stability. Bacteriocin IW1 neutralized few strains of *V. vulnificus*, BC1 eliminated several strains of *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* and, finally, BC2 neutralized *Vibrio* spp. *Plesiomonas shigelloides* and *E. coli*. Taking into account both the broadest inhibitory spectrum for *Vibrio* spp. and bacteriocin stability, BC2 was proposed as a new method of control of *Vibrio* spp. (Shehane & Sizemore, 2002).

Bacteriocins have been also investigated as an alternative solution to contamination by *Listeria monocytogenes*. A large number of IIa class bacteriocins were proposed as highly active against these bacteria (Riley & Wertz, 2002).

Bacteriocins have numerous applications as controlling agents in food but the US FDA only recognizes some bacteriocins as safe for the production of fermented foods such as Nisin, a bacteriocin produced by lactic acid bacteria (Riley & Wertz, 2002; Shehane & Sizemore, 2002). Despite their relatively narrow spectrum of activity against specific bacterial pathogens, bacteriocin's use for the preservation of food creates the dilemma of selecting resistant strains or cross-resistant strains (Riley & Wertz, 2002).

Naturally occurring bacteriophages have been used as biocontrol agents in aquatic environments for fish diseases and other infections (Nakai & Park, 2002). It has been suggested that phage treatment could be useful in controlling *Vibrio splendidus* infection (Sugumar *et al.*, 1998) in cultured larvae of the Pacific oyster (*Crassostrea gigas*) (Park & Nakai, 2003). Berthe (2005) suggested bacteriophages for the treatment of bacterial

infections in molluscan aquaculture production (Berthe, 2005). Although these reports focus on bivalve's pathogens, a similar application could be given to human pathogens. However, reports on microbial control with phages are not available for any bivalve specie or bacterial infection.

Due to the drawbacks associated with obtaining edible shellfish, additional post-harvest processing methods are also being investigated as an alternative for ensuring shellfish safety for human consumption. Since 1992, high pressure processing (HPP) has been proposed as a physical method for food preservation and has already found several commercial food applications, including oyster processing (Murchie *et al.*, 2005). HPP technology makes the inactivation of numerous microorganisms possible by exposing molluscan shellfish to relatively high hydrostatic pressure, for a short period of time at ambient temperatures, while retaining the raw taste, appearance, texture and nutritional properties of the raw shellfish. The same process can be used for shucking oysters without any mechanical force (Kingsley *et al.*, 2007). These characteristics favour both the shellfish processing industry and consumers. Even though HPP treatment offers advantages over conventional processing techniques in enhancing food safety, the protection is dependent on the composition of food and on the target microbiota. Microorganisms can differ widely in their intrinsic sensitivities to HPP (Murchie *et al.*, 2005). There is experimental evidence that *Vibrio vulnificus*, *V. parahaemolyticus*, and *V. cholerae* are reduced by HPP (Calci et al., 2005; Murchie et al., 2005). However, other bacteria reveal a wide range of resistance to HPP depending on the strain (Gram-negative bacteria are, generally, more susceptible than Gram-positive species), growth phase, growth temperature and the composition of surrounding matrices (Murchie *et al.*, 2005). Reports on the use of HHP treatment on raw shellfish showed a reduction of infectious HAV (Calci *et al.*, 2005). However, similarly to bacteria, viruses also differ widely in their vulnerability to HPP (Murchie *et al.*, 2005). Algal toxins will probably be less affected by HPP, but further studies are needed.

The efficiency of HPP-inactivation of microorganisms in shellfish needs further investigation that must include different internal locations of bacteria and viruses in the bivalve, the seasonal and geographical variations in shellfish physiology and composition and lastly the isotonic strength of the harvest waters. Also, additional investigation is needed to determine the mechanisms of inactivation, the reason for the different resistance

of viruses and the potential hazard of sub-lethally injured microbiota that may recover and multiply during subsequent storage and may lead to an over-estimation of microbial inactivation. The effects of HPP on both microorganisms and seafood are highly dependent on processing parameters that also need further investigation (Murchie *et al.*, 2005).

In contrast to the previously mentioned, porphyrins present a distinct way of improving shellfish quality since it is focused on the reduction of water contamination rather than in the bivalve. Porphyrins are compounds of natural origin which, when irradiated, generate some hyper-reactive and highly cytotoxic oxygen species (mainly, singlet oxygen) attacking different cellular components. Recently, porphyrins were synthesized to attack several types of microbial cells. The irradiation of the porphyrin causes mortality of a variety of pathogenic agents including Gram-positive and Gram-negative bacteria and parasites in either the cystic or the vegetative stage. These compounds were pointed as a novel photochemical technique for the treatment of microbiologically polluted aquaculture waters (Magaraggia *et al.*, 2006).

7. CONCLUSIONS

The nutritional and economical value of shellfish is acknowledged worldwide. Similarly, filter-feeding bivalves are well known as efficient transmitters of seafood-born disease. Over a long period of time, the high-risk nature of this product and the underestimation factors, have been well documented in many investigative reports and international agencies.

Preventive measures to enhance the quality of living bivalve shellfish when commercialized have included the monitoring and improvement of the water quality found at the harvesting areas. Nevertheless, bacterial indicators used for shellfish health evaluation were announced, in different reports, as inadequate predictors of the presence of autochthonous bacteria and human enteric viruses. Considering the results of these findings, in order to ensure public health, more accurate indexes of water quality and bivalve microbiological safety are required since they are still not available. Also the predictive value of putative indicators needs further evaluation, as specific disadvantages and contradictory results in their use have been pointed out by past studies. Indeed, the overwhelming findings of these studies suggest that the potential indicators may

complement the use of *E. coli* for a better guarantee of sanitary safety. However, the development of a local diagnostic scheme for direct detection and identification of the existing pathogens for monitoring bivalve health is probably a future tendency. Future investigations should address the relationships between indicator microorganisms survival with regard to that of the pathogens they are designed to predict. Further work is required to establish a scientific agreement among those considered potential indicators, or others to be discovered, and also to understand the implications of their introduction into legislation. Different threshold levels necessary to cause illness between pathogenic bacteria and viruses must also be considered.

Conventional methodology, applied to predict the level of contamination by quantifying bacterial indicators, needs to be improved in specificity and reduced in time. Detection by new molecular methods may be more sensitive and specific, which will allow for a faster response to health safety problems. The adjustment of the threshold levels of contamination for bacteria and viruses in relation to the risk of occurrence of disease must also be considered. Methods of detecting several pathogens should be implemented so that the assessment of microbial contamination can be more closely associated with the results produced by epidemiological studies.

Depuration and relaying helps to improve shellfish quality but if prevention of human or animal-induced pre-harvest contamination can be achieved, natural causes will always be present. A better knowledge of the parameters affecting the kinetics of the processes of depuration is still needed. More sensitive, reliable, and universally accepted depuration procedures must be developed, so that standardized methodologies can enable the comparison between the experimental results. Technological advances should also be employed.

Reoccurrence of seafood-borne diseases lead to the investigation of alternative methods to eliminate microbial contamination. Bacteriocins, bacteriophages, HPP and porphyrins may be future approaches to control shellfish microbiological contamination. The increased use of antibiotics for the treatment of disease has lead to the emergence of multiresistant bacteria, which can be released to the environment re-entering the food chain, and consequently, represent a higher risk to consumers. Particular attention should be given to multi-resistant pathogenic bacteria in order to ensure that present or new indicators will be

correlated with pathogen occurrence and that methodologies assure the elimination of these bacteria.

Consumer protection involves both the knowledge of the risk associated to the ingestion of raw shellfish and the preventive actions that take into account shellfish specificity, shellfish contamination and adequate regulations. The combination of new depuration approaches and a more accurate quality assessment will help to relieve public concern regarding foodborne diseases associated with shellfish products.

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Chapter III

Modified methodology for the extraction of bacterial DNA from mussels – relevance for food safety

Modified methodology for the extraction of bacterial DNA from mussels – relevance for food safety

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ABSTRACT

The control of the microbiological quality of bivalve molluscs assumes particular importance because they are among the most produced seafood products, and mostly consumed as a whole, raw or lightly cooked. The composition of the bacterial community associated to bivalves depends mostly on the microbiology of the surrounding environment at growing sites. Once the relationship between microbiology of bivalves and environment is established, a better classification and monitoring of the shellfish beds and evaluation of depuration strategies can be achieved. In this work we tested if the methods of DNA extraction commonly used for the culture-independent microbiological analysis of sediment and water could be used directly, or with modifications, for the analysis of bacteria in mussels.

The commercial kits Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), UltraClean™ Soil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, California) and the method described by Griffiths and collaborators (2000) for DNA/RNA co-extraction were compared. The efficiency of extraction was assessed by DNA fluorescence and the DGGE gel patterns of 16S rRNA gene fragments were used to compare the reproducibility and representativeness of the extraction methods. Results showed that the DNA/RNA co-extraction method with modifications was the most suitable. However, the results must be interpreted in the light of the purpose of the study and the relevance of maximizing extraction yield or diversity estimate, without compromising reproducibility.

To our knowledge, this was the first attempt to transpose the procedure currently used for DNA extraction from sediments or waters, to the analysis of whole mussels.

KEYWORDS: Bacterial DNA extraction; Mussels; DGGE; Food safety.

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1. INTRODUCTION

Molluscan shellfish are the second largest aquaculture product worldwide, and the blue mussel (*Mytilus edulis*) is among the most widely produced species (FAO, 2010).

Bacterial contamination is one of the main constrains to this economic activity (Huss *et al.*, 2004). By filter-feeding nutrients from the surrounding environment, bivalves bioaccumulate autochthonous or anthropogenic bacteria, being extremely susceptible to contamination (WHO, 2010). Considering that the traditional cooking procedure of bivalves (lightly cooked) may not completely ensure the inactivation of microbes or toxins, bivalves are critical items in terms of food safety (Lees, 2000; Romalde *et al.*, 2002; Murchie *et al.*, 2005). Therefore, the control of the microbiological quality of these products and the classification of growing areas according to sanitary quality (Anonymous, 2010) assumes particular importance (Pinto *et al.*, 2006). The presence of indicator bacteria (*Escherichia coli*) in concentrations above microbiological standards in bivalve flesh and intravalvular liquid (FIL) may predict the presence of pathogenic bacteria (Anonymous, 2004a, b, 2005, 2007, 2008), which implies that the product does not meet the requirements for human consumption and triggers the interdiction of growing areas with consequent economic losses to this sector of activity (IPIMAR, 2008). Also, the decision on the need of depuration procedures, prior to commercialization, is based on the levels of contamination at the growing beds (Oliveira *et al.*, 2011).

Conventional methodology applied to assess microbiological quality of bivalves involves mainly cultivation procedures, using enrichment broths followed by the isolation of colonies on selective media, and confirmation with differential media and biochemical tests (Gugliandolo *et al.*, 2010). Although useful, these techniques are laborious, time consuming and susceptible of producing false-positives (Ward *et al.*, 1990; Wagner *et al.*, 1993; Hugenholtz *et al.*, 1998; Hernández-Zárate & Olmos-Soto, 2006; Oliveira *et al.*, 2011). In recent years, 16S rRNA gene sequences have been extensively used for detection, identification and quantification of bacteria in different samples, namely environmental matrices and food products, by PCR-based methods (Thompson *et al.*, 2005; Hernández-Zárate & Olmos-Soto, 2006). These approaches have often the advantage of being more rapid, sensitive and specific, being independent of bacteria metabolic activity, culturability or pathogenicity (Rompré *et al.*, 2002). These advantages are particularly interesting when risk assessment is conducted in the perspective of human

health. Considering that the first signs of illness often occur in less than 24 h after ingestion of contaminated seafood, a rapid response is required so that the cause can be established, geographically localized and the occurrence of outbreaks can be efficiently prevented (Gugliandolo *et al.*, 2010; Oliveira *et al.*, 2011).

Taking into account that filter-feeders accumulate bacteria from the surrounding environment, the parallel monitoring of the bacterial communities in the sediments, water and in whole molluscs would provide a more informative approach. Several methods for the extraction of bacterial DNA from sediments and water for metagenomic analyses are currently available in scientific literature. However, to our knowledge, the applicability of these procedures to the extraction of microbial DNA from whole molluscs in order to obtain information susceptible of comparative analysis of the associated bacterial communities has not been tested.

We intended to assess which culture-independent approach of DNA extraction could be used to analyze bacterial communities associated to whole mussels allowing future comparison of their microbial communities to those of sediment and waters. Robust DNA extraction procedures commonly used for the analysis of complex microbial communities in sediment/water samples were adapted and compared for the extraction of microbial DNA from whole mussels, taken as model-bivalves for this study. The results were analyzed in the perspective of achieving good extraction yields, high reproducibility and the widest possible representativeness of bacterial diversity.

2. MATERIALS AND METHODS

2.1. SAMPLING SITE AND COLLECTION OF MUSSELS

The study area was Ria de Aveiro, a multi-estuarine ecosystem located on the Northwest coast of Portugal (Figure 1). Pollution of Ria de Aveiro is mainly derived from diffuse drainage of domestic sewage and to harbor and industrial activities, aquaculture farms and run-off from agriculture fields (Henriques *et al.*, 2004). For this study, *Mytilus edulis* (blue mussels) were collected from Ílhavo Channel (40°35'17"N, 8°41'9"W). This location was chosen because it represents the largest persistent and highly exploited area of mussel cultivation within the estuary (Figure 1) and it is chronically affected by natural-occurring

and anthropogenic microbial contamination being classified as a C zone (]4600; 46000] *E.coli*/100g FIL) (Campos & Cachola, 2006; IPIMAR, 2008). Mussel specimens were hand collected with the aid of a small rake, transferred to sterile bags and refrigerated at 4 °C during transport to the laboratory. Analyses were conducted within 3 h after collection.

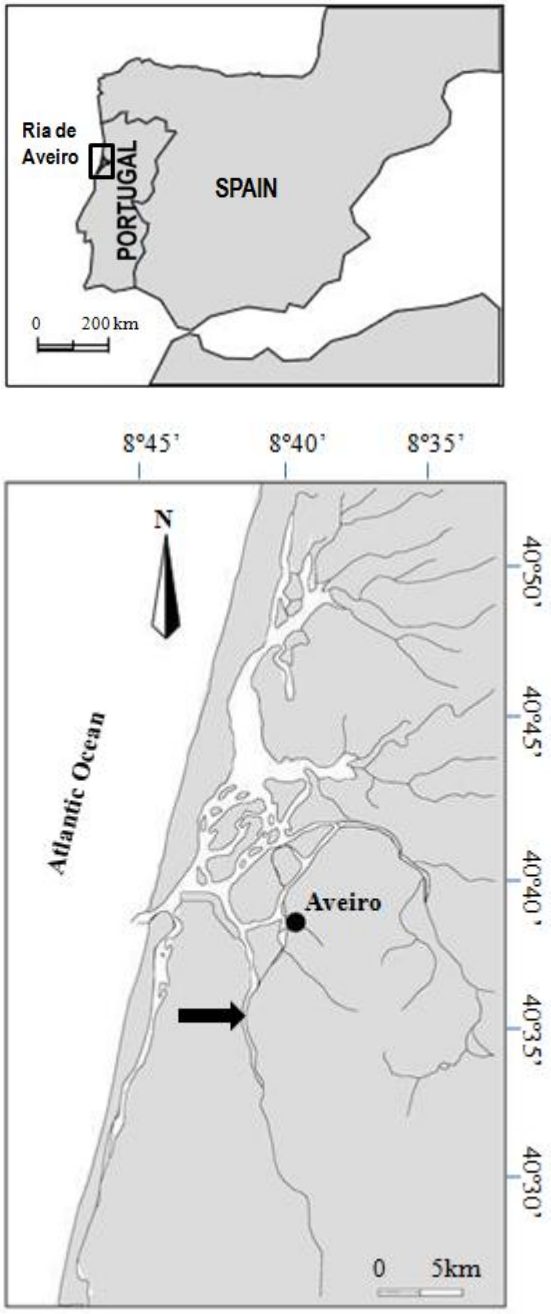

Figure 1 *Ria de Aveiro* lagoon (Portugal) with sampling site indicated by an arrow (Adapted from Henriques *et al.*, 2004).

2.2. MUSSEL PREPARATION

The shells of intact mussel specimens were scrubbed, washed in tap water and thoroughly rinsed with Mili-Q sterilized water. Mussels shells were aseptically opened and a total of 25 g of FIL were removed and collected on a sterile beaker to which a volume of 250 ml of sterile 8.5% saline solution (Sigma-Aldrich, Co., St. Louis, MO) (1:10) was added. The mixture was homogenized with a Sterilmixer 12 (PBI international, Milano, Italy) at 11 000 rev min^{-1} for 20 s (Croci *et al.*, 2001). This homogenate was used for all the extraction treatments. Replicates of 30 ml were collected after agitation of the homogenate. Triplicates for each method were randomly chosen from the bacth of 30-ml replicates. Homogenates were centrifuged in a Beckman Avanti^{TM} Centrifuge J25I with rotor JA-25.50 (Beckman Coulter, Fullerton, CA) at 1 000 rpm for 5 min for removal of fragments of mussel tissues. The supernatants (25 ml) were centrifuged at 10 000 rpm for

20 min in a Beckman Avanti^{TM} Centrifuge J25I with rotor JA-25.50 (Beckman Coulter, Fullerton, CA) for bacterial biomass precipitation and the liquid phase was discarded. The pellets were resuspended in 1.5 ml of 96% ethanol (Sigma-Aldrich, Co.**,** St. Louis, MO) for preservation until later DNA extraction, PCR amplification and Denaturing Gradient Gel Electrophoresis (DGGE) analyses.

2.3. DNA EXTRACTION

For DNA extraction, the material preserved in ethanol was collected by centrifugation at 13 000 rpm for 15 min using a Sigma 1-14 Centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany). DNA was then extracted by three distinct methods: (a) with the commercial Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), usually used for water and tissue matrices, used according to Henriques and collaborators (Henriques *et al.*, 2004); (b) by the DNA/RNA co-extraction method proposed by Griffiths and collaborators, commonly used for sediment matrices; (c) with the commercial UltraClean^{TM} Soil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, California). These extraction methods were selected envisaging the possibility of their parallel application to mollucs, water and sediments from the growing areas allowing future comparison of the corresponding bacterial communities.

2.3.1. DNA EXTRACTION WITH GENOMIC DNA PURIFICATION KIT

The pellet (0.1 g) was resuspended in 400 µl TE buffer containing 10 mg ml^{-1} of lysozyme (Sigma-Aldrich, Co.**,** St. Louis, MO) followed by incubation for 1 h at 37 ºC in order to achieve chemical disruption of bacterial cells (Henriques *et al.*, 2004). DNA was extracted and purified using the commercial Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), according to the instructions of the manufacturer. DNA was dissolved in 50 µl Tris-EDTA buffer (pH 7.4) (Sigma-Aldrich, Co.**,** St. Louis, MO) and stored at -20 ºC until further use. DNA obtained by this method will be hereafter referred as F-DNA.

2.3.2. DNA/RNA CO-EXTRACTION METHOD

Nucleic acids (DNA and RNA) were extracted from 0.1 g of pellet according to Griffiths and collaborators (Griffiths *et al.*, 2000). DNA obtained by this method will be hereafter referred as G-DNA.

2.3.3. DNA EXTRACTION WITH ULTRACLEAN™ SOIL DNA ISOLATION KIT

DNA was extracted from 0.1 g of pellet with the UltraClean™ Soil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, California) following the instruction of the manufacturer and will be hereafter referred as M-DNA.

2.3.4. MODIFICATIONS ON THE PREPARATION OF MUSSEL MATERIAL PRIOR TO THE DNA EXTRACTION METHODS

The F-DNA, G-DNA and M-DNA methods were also performed after introducing some initial modifications which intend to improve cell lysis.

The pellet (0.1 g) was resuspended in 400 µl TE buffer and submitted to physical treatment by imposing six freeze-thaw cycles (40 seconds at -196 ºC with liquid nitrogen and 2 min at 55 °C with dry heat) and other six cycles of freeze-thawing including 3 min of ultra sounds for each cycle. For G and M methods, the pellet (0.1 g) was collected by centrifugation at 13 000 rpm for 15 min using a Sigma 1-14 Centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany) for further extraction. Extraction of nucleic acids was than performed as previously mentioned. DNA obtained using these modifications prior to the respective extraction method will be hereafter referred as m-DNA.

2.4. DNA PURIFICATION

Nucleic acids obtained by the different procedures were purified with the GENECLEAN® SPIN Kit (Qbiogene, Inc., Carlsbad, California) according to the instructions of the manufacturer in order to remove impurities and reagents introduced during extraction, prior to downstream uses of the extracted DNA.

2.5. DNA QUANTITY AND PURITY

DNA quantity and purity were assessed with the NanoDrop® ND-1000 v 3.3 spectrophotometer (NanoDrop Technologies, Inc, Wilmington, Delaware USA). Alternatively, the DNA quantity was determined by using the Qubit® Fluorometer (Invitrogen™, Life Technologies™, UK) with the Qubit® dsDNA BR Assay Kit (Invitrogen™, Life Technologies™, UK) according to the instruction of the manufacturer.

2.6. AMPLIFICATION OF 16S RIBOSOMAL RNA GENE

The DNA extracted was used to amplify the bacterial 16S rRNA gene fragments by a nested approach. In the first PCR were used 0.25 µM of each of the universal primers 8/27f and 1492/1512r (Integrated DNA Technologies, BVBA, Munich, Germany) (Weisburg *et al.*, 1991). The PCR reaction mixture contained 1 µl of template DNA, 3.75 mM $MgCl_2$ (MBI Fermentas, Vilnius, Lithuania), 0.2 mM deoxynucleoside triphosphates (MBI Fermentas, Vilnius, Lithuania), 1 X PCR Buffer (MBI Fermentas, Vilnius, Lithuania), and 1 U Taq polymerase (MBI Fermentas, Vilnius, Lithuania). Reaction was performed in a MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Denaturation of the template for 5min at 94ºC was followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 2 min at 72°C. The final extension lasted 10 min at 72ºC.

For DGGE analysis, a 410-bp rRNA gene fragment was amplified with primers F968-GC-clamp and R1401 (Integrated DNA Technologies, BVBA, Munich, Germany) (Nübel *et al.*, 1996) using as template 1 µl of the product obtained from the first PCR. Amplification was conducted in a MyCycler™ thermal cycler (Hercules, California, USA). Additionally, the reaction mixture contained, 4% acetamide (Sigma-Aldrich, Co., St. Louis, MO), 0.2 mM deoxynucleoside triphosphates P (MBI Fermentas, Vilnius, Lithuania), 1X PCR Buffer (MBI Fermentas, Vilnius, Lithuania), 0.1 µM of each primer, 3.75 mM $MgCl_2$ (MBI Fermentas, Vilnius, Lithuania) and 1 U Taq polymerase (MBI Fermentas, Vilnius, Lithuania). After 4 min of initial denaturation at 94°C, 30 cycles of 1 min at 94°C, 1 min at 53°C and 1.30 min at 72°C. Finally, an extension step at 72°C for 10 min was carried. Negative and positive controls were included in all reactions.

2.7. DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) ANALYSIS

DGGE was performed with the DGGE-2401 system (C.B.S. Scientific Co., Del Mar, CA, USA). PCR amplification products were loaded onto 6.5% acrylamide gels using a denaturing gradient ranging from 22 to 58%, where 100% denaturant corresponded to 7 M urea (VWR International, LLC, Radnor, Pennsylvania) and 40% (vol/vol) formamide (Sigma-Aldrich, Co., St. Louis, MO). A marker composed by 11 bands was included in the extremities of each gel (Heuer *et al.*, 2002). The electrophoresis was performed at 58°C for 6 h at 220 V in 1X TAE buffer (5 Prime, Hamburg, Deutschland). After electrophoresis, the gels were stained and fixed for 20 min with 0.3 g silver nitrate (Sigma-Aldrich, Co., St. Louis, MO) in 0.1% (v/v) ethanol 96% (Aga S. A., Loures, Lisboa) and 0.005% acetic acid (Merck KGaA, Darmstadt, Germany), rinsed and submerged in a developing solution of 0.003% (v/v) formaldehyde 37% (Sigma-Aldrich, Co., St. Louis, MO) and 0.33% sodium hydroxide (9%) (Merck KGaA, Darmstadt, Germany). Finally, 0.75% Na_2CO_3 was added to stop the development and the gel was scanned using a Molecular Image FX apparatus (Bio-Rad, Hercules, California, USA).

2.8. ANALYSIS OF DGGE PATTERNS AND STATISTICAL ANALYSIS

The analysis of the DGGE gel was done using Gelcompar 4.0 program (Applied Math, Ghent, Belgium) software package (Smalla *et al.*, 2001). The band profiles from each treatment were exported to a spreadsheet as previously described (Gomes *et al.*, 2010). The band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane, construing a binary matrix. Analysis of DGGE profiles was performed using the PRIMER v5 software (Primer-E Ltd., Plymouth, UK) (Clarke & Gorley, 2001, 2006). In the DGGE profiles, the number and precise position of the bands were used as an estimation of the number of different ribotypes present in the community (Muyzer *et al.*, 1993; Miller *et al.*, 1999). DGGE patterns were examined using the Shannon–Weaver index of diversity, *H' (Shannon & Weaver, 1963),* calculated as follows: $H' = -\sum(n_i/N)\ ln(n_i/N)$, where n_i is the relative surface intensity of each DGGE band and N is the sum of all the surfaces for all bands in a given sample. Statistical significance of variance of the means of the Shannon-Weaver index was evaluated with a one-way

analysis of variance (ANOVA). The binary matrix was then transformed into a similarity matrix using the Bray-Curtis similarity index considering band position and intensity. Multivariate analyses of DGGE profiles using this similarity matrix included: (1) cluster analysis, obtained by grouping each DGGE profile according to their similarity (Ramette, 2007); (2) analysis of similarities (ANOSIM) tested using 999 permutations, which aimed to determine differences between the treatments compared to variation within treatments, testing the null hypothesis that there are no differences (R=0) (*R test* value generally ranges from 0 to 1, where higher values correspond to higher differences: $R \leq 0.25$ - no significant differences, $0.25 < R < 0.50$ - low significance, moderate separation and $0.50 < R \leq 1.00$ - high significance, high similarity (Chapman & Underwood, 1999; Clarke & Gorley, 2001; Ramette, 2007); (3) multidimensional scaling (MDS) diagram, a two-dimensional map with artificial *x*- and *y*-axis, was constructed so that each DGGE profile is placed as one point and similar profiles are plotted together (stress values range from 0 to 1, revealing the reliability of the results for lower values) (Ramette, 2007).

3. RESULTS AND DISCUSSION

3.1. DNA EXTRACTION YIELD

Underlying the objectives of this work was the assumption that if the same method of extraction of bacterial DNA could be used in molluscs, sediments and water of harvesting/production sites, a very straightforward assessment of the microbial loads of these areas could be rapidly achieved, also providing the basis for the decision on eventual classification of harvesting areas and depuration strategies. For that, protocols currently used for extraction of nucleic acids from sediment, water and animal tissues were tested with and without specific modifications. Whole mussels were used as samples for a direct extraction considering that bivalves are eaten as a whole. Bioaccumulation of some pathogens may occur especially in the digestive tube (Power & Collins, 1990) but they may also extend to other tissues and be infectious even in very low numbers, constituting a higher risk to public safety (Richards, 1988; Lees, 2000). Also, this approach is less laborious because dissection is not required. To our knowledge this is the first attempt to extract bacterial DNA from an entire bivalve.

DNA yields corresponding to the different extracted protocols tested in this work are summarized in Table 1. The yield of DNA extraction by mF method was the highest by both UVQ and fluorescence-based quantification (FQ). However, some DNA from the blue mussel may have been contaminated bacterial DNA. This effect may also be present in the modified methods because of the additional mechanical disruption of bivalve tissues (Joanne *et al.*, 1995). The yield of DNA extraction inferred from the fluorescence-based quantification decreased according to the following order: mF, F, mG, G, mM and M method. Higher values were obtained by extraction methods including the modification corresponding to the preliminary freeze-thaw sequence.

Table 1 Comparison of the extraction yield of the different protocols tested for the extraction of bacterial DNA from whole-bivalve (*Mytilus edulis)* material.

Extraction protocol	DNA yield (µg/g) (*Mytilus edulis* sample)	
	UVQ[a]	FQ[b]
Fermentas Kit (F)	21.48 ± 1.22	5.69 ± 0.37
Modified Fermentas Kit (mF)	80.42 ± 0.58	27.13 ± 0.62
MOBIO (M) Kit	0.73 ± 0.59	0.23 ± 0.10
Modified MOBIO (mM) Kit	4.58 ± 1.43	1.82 ± 1.41
DNA/RNA co-extraction (G)	8.75 ± 0.32	4.97 ± 0.17
Modified DNA/RNA co-extraction (mG)	9.55 ± 0.60	0.47 ± 0.30

[a]DNA yield based on NanoDrop® quantification.

[b]DNA yield based on Qubit® dsDNA BR Assay Kit quantification.

3.2. DNA PURITY

The UV absorbance (A260/A280 ratio) was used to assess DNA quality (Figure 2). The deviation from the theoretical value of approximately 1.8 for DNA extracts indicates the presence of protein, phenols or contaminants absorbing strongly in the 280 nm region. The extracts obtained by the tested protocols presented a range of 280/260 ratios of 0.93 to 2.09 (Figure 2) indicating some contamination with RNA in the mF treatment, and with protein or phenol in the treatments M and mM. It has been reported that beadbeating, PEG

precipitation and CTAB treatment steps generally improve DNA purity (Cullen & Hirsch, 1998; Lai *et al.*, 2006). These steps are included in the DNA/RNA co-extraction method (G) which, according to the 280/260 ratio, produces the highest level of purity among the tested procedures.

PCR is a highly sensitive, specific and rapid method for detecting bacterial gene sequences in pure cultures and natural waters. However, when applied to food samples, the amplification reaction can be inhibited or its sensitivity reduced severely (Joanne *et al.*, 1995) by the presence of compounds from the food matrix. Therefore, in addition to quantity, the quality of the retrieved DNA is of major importance for the application of downstream methods of molecular microbiological analysis.

PCR reaction using templates of DNA obtained by the different methods were not consistent in the degree of amplification. In fact, amplification failed for F-DNA and for one of the replicates of mF-DNA (mF-c), although both methods achieved a good extraction yield and only a slight contamination of mF-DNA with RNA could be inferred from the A280/A260 ratio. This may indicate the presence of other PCR inhibitors (Rossen *et al.*, 1992) in the extracts obtained by the F or mF procedures.

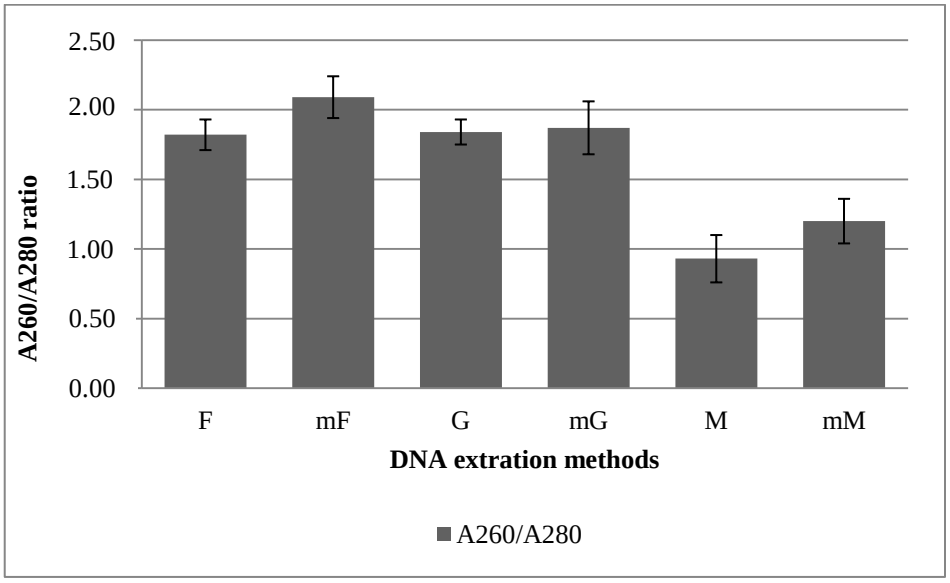

Figure 2 Ratio A260/280 as an estimate of the purity of DNA obtained by three different extraction methods, with and without the initial freeze-thaw and ultra-sound modification. The values are expressed as the average of three measurements. Error bars represent the standard deviation.

3.3. REPRESENTATIVENESS OF THE BACTERIAL DNA EXTRACTS

DGGE gel patterns were used for the comparison of the three extraction methods and corresponding modifications used to obtain bacterial DNA from whole mussels. The DGGE profiles are represented in Figure 3.

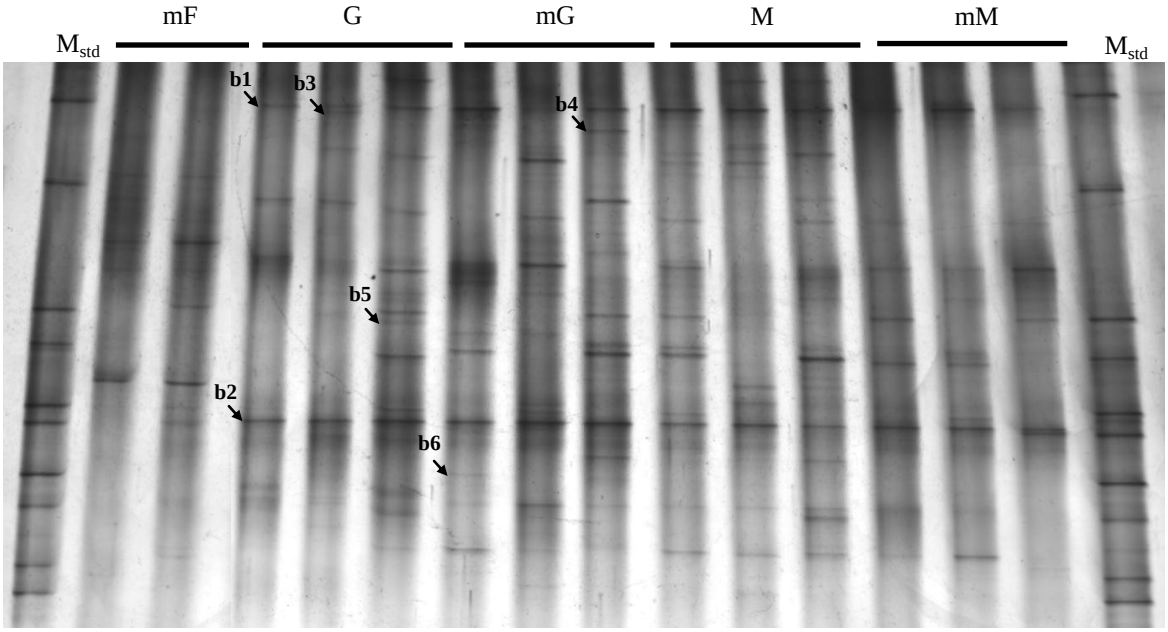

Figure 3 DGGE profiles of the bacterial community associated to *Mytilus edulis* using different techniques for the extraction of bacterial DNA. DGGE profiles of F-DNA and of replicate mF-c are not presented due to failure in amplification. In the figure: M_{std}—marker; F—DNA extraction method with Genomic DNA Purification Kit; G—DNA/RNA co-extraction method proposed by Griffiths and collaborators; M—DNA extraction method with the UltraClean™ Soil DNA Isolation Kit; m—extraction methods including the modification corresponding to the preliminary freeze-thaw sequence. Replicates of the same extraction method are ordered as follows: a, b, c (a, b for mF).

Differences in DGGE band patterns were clearly visible for the different extraction methods. The number of bands per lane ranged from 9 to 25 in the mF and mG treatments, respectively. Some bacterial ribotypes, represented as dark bands, could be identified in all treatments (*e.g.* bands 1 and 2) and were interpreted as representing highly abundant bacteria. Other bacterial ribotypes (*e.g.* bands 3, 4, 5 and 6) were only present in the extracts corresponding to some of the treatments and most samples produced higher numbers of weak bands, near the limit of detection, which were assumed to represent less abundant ribotypes. The bivalve acts as a colonizing area for the microorganisms present in

the immediate environment, in which diversity may vary considerably within very small spatial scales (Gillan *et al.*, 1998). Also, low intensity DGGE bands may represent less abundant 16S rRNA sequences that may be more affected by extraction and PCR biases than the common and abundant sequences.

The diversity of rybotypes in each DGGE profile was estimated by using the Shannon-Wiener index (H') presented in Table 2. The DNA yield and quality could explain to some extent, the diversity of the bacterial community represented in the DNA extract. DNA obtained with the F protocol could not be amplified and mF method produced the lowest diversity of rybotypes (*H'*=1.965). The estimated values of bacterial community diversity were highest for mG-DNA. Differences of the diversity of rybotypes obtained by each protocol were not significant, F (4, 9) = 2.159, p =0.155 (Table 3).

Table 2 Shannon–Weaver index of diversity (*H')* for each replicate of the different extraction methods.

Sample	Diversity (*H'*)	*H'* Mean	*H'* Standard deviation
mF-a	1.777	1.965	0.265
mF-b	2.152		
G-a	2.320		
G-b	2.381	2.560	0.365
G-c	2.980		
mG-a	2.370		
mG-b	2.967	2.664	0.299
mG-c	2.654		
M-a	2.488		
M-b	2.207	2.489	0.283
M-c	2.773		
mM-a	2.417		
mM-b	2.467	2.446	0.026
mM-c	2.454		

In the table: F—DNA extraction method with Genomic DNA Purification Kit; G—DNA/RNA co-extraction method proposed by Griffiths and collaborators; M—DNA extraction method with the UltraClean™ Soil DNA Isolation Kit; m—extraction methods including the modification corresponding to the preliminary freeze-thaw sequence; a, b and c—replicates of the same extraction method.

Table 3 One-way analysis of variances (ANOVA) of the means of Shannon–Weaver index of diversity (*H'*).

	Sum of Squares	Degree of freedom	Mean Square	*F*	*p*-value	*F* critic
Between treatments	0.649	4	0.162	2.159	0.155	3.633
Within treatments	0.676	9	0.075			
Total	1.325	13				

Cluster analysis defines groups of DGGE profiles with similar banding pattern/community structure and was used to verify the similarity among replicates of the each extraction methods. The dendrogram representing the cluster analysis (Figure 4) shows a high degree of variability among replicates. Only the three replicates of the mM method grouped adjacently, with no more than 53% similarity among them. The similarity of the three replicates of the M protocol was 39%, while for the G and mG protocols was only 11%. The two replicates of the mF protocol that could be amplified showed a similarity of 55%. If the presence of high numbers of very weak bands in the DGGE patterns is interpreted as an indication of a community structure where the majority of the frequent rybotypes are present with abundances very close to the detection limit, the modest similarity between replicates may be a consequence of the random presence or absence of a rybotype in each particular replicate.

Considering that dendrograms do not fully represent all pairwise similarities between DGGE patterns, analysis of similarities (ANOSIM) and multidimensional scaling (MDS) were used to refine the perspective on the similarity between replicates of each procedure and between procedures. The ANOSIM (Table 4) demonstrated that here are significant differences among profiles obtained from each extraction protocols (global R=0.641). MDS analyses (Figure 5) corroborate ANOSIM results, indicating that the DGGE profiles corresponding to extracts obtained with the mF method are clearly separated from those obtained with all other treatments (R=1.0). The R value of the pairwise analysis of group mG and G method was negative (R=-0.148) due to the fact that G method contained an outlier replicate (the G-c replicate, as observed in the MDS plot) showing lack of positive correlation between the different replicates (Chapman & Underwood, 1999). Indeed, the DGGE profile of the replicate G-c showed a higher diversity (*H'*=2.980) within the G method (Figure 3 and Table 2). Furthermore, G replicates showed the lowest similarity

(11%) of all the tested protocols (Figure 4). The DGGE profiles of mG and M protocols did not show significant differences (R=0.148), implying higher similarities within replicates patterns when compared to similarities between methods. In MDS plot, for the G and M protocols, the patterns corresponding to the freeze-thaw modifications are more similar (R=0.556) than the respective non-modified procedures (R=0.593), which clearly produced different banding patterns. This result shows that the freeze-thaw modification enabled the extraction of more common ribotypes in M and G methods. Considering that the freeze-thaw modifications improved bacterial DNA extraction yield from the mussel matrice, the enhanced similarity between mM and mG protocols may results from a better recovery of the more rare rybotypes which are close to the detection limit of the technique and that could easily be missed in some replicates of the standard M or G protocols making the DGGE patterns less reproducible.

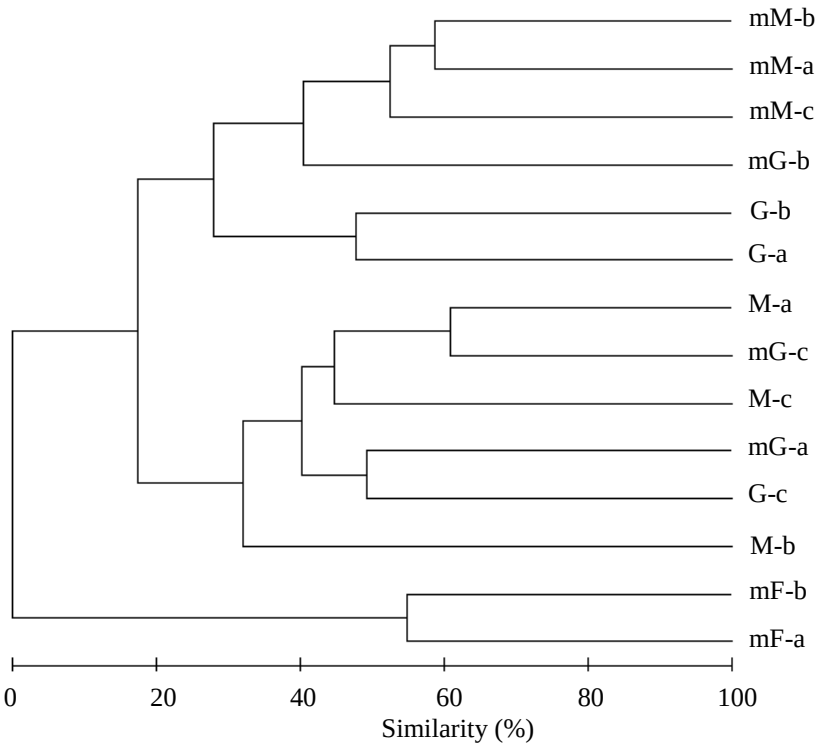

Figure 4 Dendrogram generated from the patterns of bands obtained by DGGE (Figure 3). In the figure: F—DNA extraction method with Genomic DNA Purification Kit; G—DNA/RNA co-extraction method proposed by Griffiths and collaborators; M—DNA extraction method with the UltraClean™ Soil DNA Isolation Kit; m—extraction methods including the modification corresponding to the preliminary freeze-thaw sequence; a, b and c—replicates.

Table 4 ANOSIM statistics analysis (R) of Bray-Curtis similarity measures generated from the patterns of bands obtained by DGGE (Figure 3) of DNA extraction method with Genomic DNA Purification Kit (F), DNA/RNA co-extraction method proposed by Griffiths and collaborators (G) and DNA extraction method with the UltraClean™ Soil DNA Isolation Kit (M).

Groups	R Statistic
mF, G	1.0
mF, mG	1.0
mF, M	1.0
mF, mM	1.0
G, mG	-0.148
G, M	0.593
G, mM	0.815
mG, M	0.148
mG, mM	0.556
M, mM	0.519
Global R	**0.641**

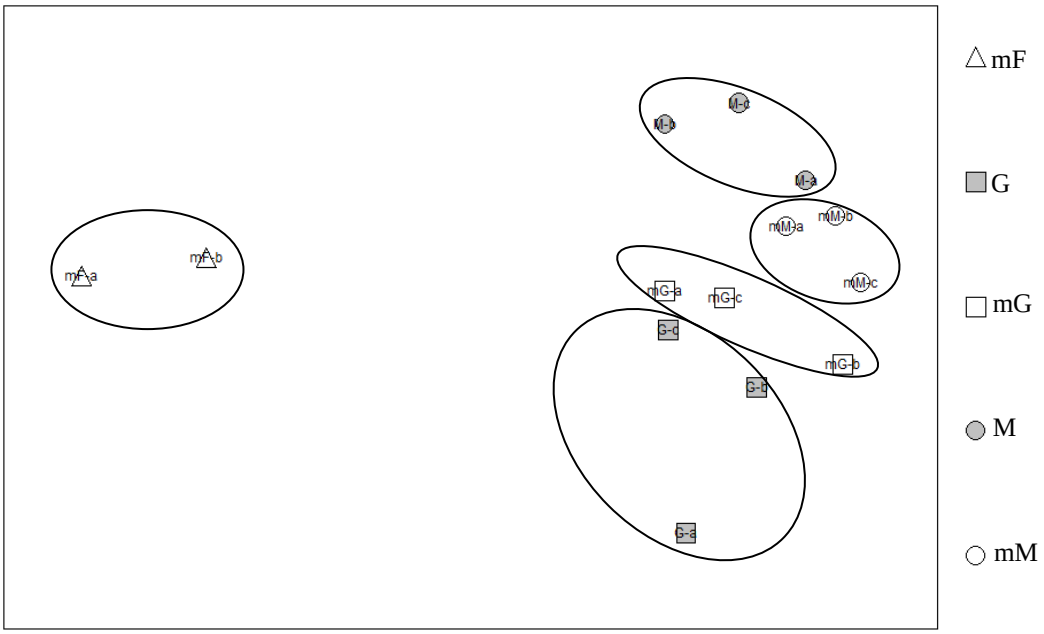

Figure 5 MDS plot (stress value of 0.13) showing relative similarity between extraction methods, as determined from the patterns of bands obtained by DGGE (Figure 3). In the figure: F—DNA extraction method with Genomic DNA Purification Kit; G—DNA/RNA co-extraction method proposed by Griffiths and collaborators; M—DNA extraction method with the UltraClean™ Soil DNA Isolation Kit; m—extraction methods including the modification corresponding to the preliminary freeze-thaw sequence.

4. CONCLUSION

The modifications applied to protocols F, G, M were effective in improving the DNA extraction yield. Some drawbacks, as the integration of non-specific inhibitors in the sample, from the body of the bivalves, must be further analyzed. The effect of sample size (mass of mussel FIL used for the extraction or particular tissues) on the reproducibility of DGGE patterns was not addressed in this work but it may be relevant in the representativeness of the bacterial DNA for metagenomic analyses. Other sample preparation techniques for PCR-based detection of bacteria in bivalves might be evaluated in the future.

The DGGE profiles were characterized by a high degree of variability between replicates which can be associated to the presence of a high number of low abundant bacteria, probably very close to the detection limit of the method. The mG method for bacterial DNA extraction was the most suitable for investigation of the bacterial community from the blue mussel *Mytilus edulis*. Despite the rather low similarity between replicates, this method showed a fair DNA extraction yield, a good degree of purity and provided the more complete representation of the community. Although being slightly more laborious because of the initial preparation of reagents, and more time consuming than the M method because of the multiple steps involved, the mG method may turn out advantageous in view of its simplicity and low cost. It is worth to consider that this approach can also be used when RNA-based analysis is intended, simply by skipping the step of DNA purification. Therefore, from the culture-independent approaches of DNA extraction here tested, mG method shows potential for the analysis of bacterial communities associated to whole mussels in the perspective of comparison of the microbial communities from bivalves, sediment and waters.

The presented research contributes to the development of a convenient approach for the classification of growing areas, assessing the microbiological quality of bivalves and shellfish growing areas by molecular methods and may ultimately provide the practical basis to routine microbiological monitoring ensuring public heath safety.

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Chapter IV

Bacteriophage therapy as a bacterial control strategy in aquaculture

Bacteriophage therapy as a bacterial control strategy in aquaculture

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ABSTRACT

Aquaculture is a sector of economic relevance worldwide. Bacterial infections have been recognized as an important limitation to aquaculture production and trade. Microbial infection in aquaculture derived products has been prevented by antibiotic administration with limited success. Recently, drug-resistant bacteria have become a global problem, urging for the prompt development of alternative control strategies in order to improve food quality and safety. The alternative approach of using lytic phages or their products, as bioagents for the treatment or prophylaxis of bacterial infectious diseases, has gained interest. This review intends to emphasize the need of further research in the field of the application of phage therapy in aquaculture and highlights the use of phages in invertebrates as an antimicrobial strategy pointing critical aspects from the economic, environmental and public health perspectives.

KEYWORDS: Bacteriophage; Bacterial infections; Aquaculture; Food safety; Public health.

*Published paper in Annexes.

1. INTRODUCTION

Aquaculture is the cultivation of aquatic populations including finfish, shellfish and plants, under controlled conditions such as breeding and confinement, along with supplying nutrients and medication, in order to increase production (Pillay & Kutty, 2005). Recently, aquaculture production has become one of the fastest-growing animal food-producing industries (FAO, 2006, 2009). Between 2004 and 2006, the annual growth rates were 6.1% (in quantity) and 11.0% (in value) (FAO, 2009). Despite the international development of this production sector, microbial diseases outbreaks resulted in important economic losses being considered the major problem associated with aquaculture (Hektoen *et al.*, 1995; Berthe, 2005). Some of the typical fish farming diseases caused by the main biological agents (bacteria, viruses, parasites and oomycetes) have been briefly reviewed elsewhere (Almeida *et al.*, 2009). Bacterial diseases caused by indigenous and non-indigenous pathogenic bacteria, particularly multiresistant bacteria, are a major issue in aquaculture. Although the administration of antibiotics is approved by authorities and it represents an easy and relative low-cost solution, if compared to the economic loss that bacterial infection causes, some important findings indicate that this strategy has limited success: development of multi-drug resistance in bacteria, microorganism substitution, ecological and public health impacts (Park *et al.*, 2000; Perreten, 2005). There is an increasing need for finding alternative ways to control microbial diseases in aquaculture. Recently, bacteriophages gained increased attention as an alternative to antibiotics and other antibacterial chemicals in order to control microbial diseases and prevent the spreading of multiresistant bacteria in aquaculture (Nakai & Park, 2002). Bacteriophages were discovered early in the twentieth century, and variable attention has been given to research on bacteriophage applications. Bacteriophages or phages are viruses that specifically interact with their host bacteria through two major infection cycles (Figure 1). These are the lytic (or virulent) and lysogenic cycles and more sporadically through pseudolysogeny (Ripp & Miller, 1997, 1998). Lytic phages replicate inside the host cell and progeny viruses are released causing cell lysis. This capacity to destroy host bacteria sets the stage for the use of lytic phages as therapeutic or prophylactic agents. A method using naturally occurring lytic phages, or their products, as bioagents for the treatment of bacterial infectious diseases is called bacteriophage therapy or phage therapy (Nakai, 2010).

This work intends to critically summarize recent literature on the application of phages in aquaculture, indentifying its benefits and potential drawbacks envisaging bacteriophage therapy as an alternative way to control bacterial diseases found in aquaculture organisms (fish, crustaceans and molluscs). We highlight the perspective of using phages in invertebrates, particularly, bivalve molluscs, providing a critical view for further research since little information concerning this antimicrobial strategy in these animals exists.

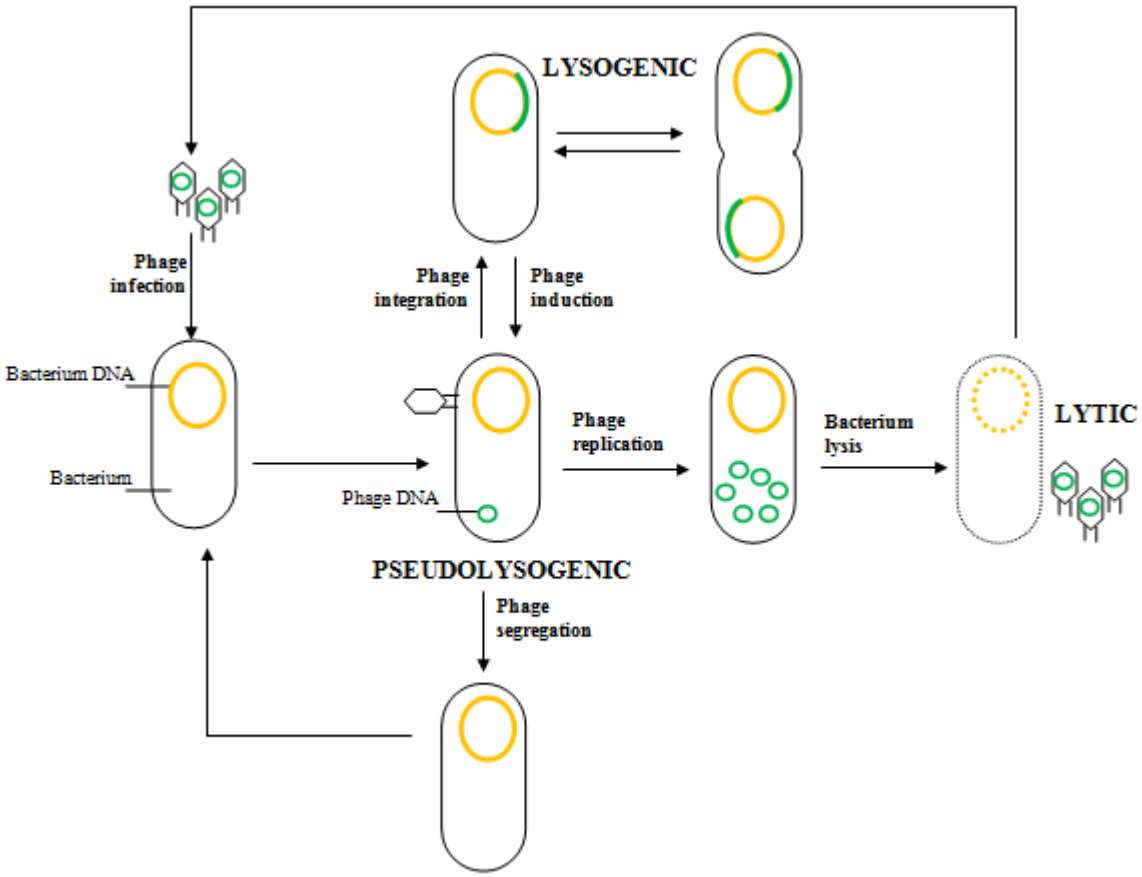

Figure 1 Phage multiplication cycles. After phage infection, and if phage segregation do not occur, temperate phages may undergo the lytic or lysogenic cycle. In the lytic cycle, new phages are produced and released during bacterium lysis. In the lysogenic cycle, the phage DNA is included in the host chromosome and replicated along with the bacterial DNA. Occasionally, the prophage may excise from the host genome and the lytic cycle begins. In pseudolysogeny infection, bacteriophage enters a dormant intracellular phase where the phage genome is not integrated into host genome. Bacteriophage can proceed to either lytic or lysogenic infection cycles (Adapted from Griffiths *et al.*, 1999; Sulakvelidze *et al.*, 2001).

2. THE NEED FOR AN ALTERNATIVE TO CHEMOTHERAPY IN AQUACULTURE

Like other animals and humans, cultured fish and shellfish are persistently colonized and infected by microorganisms (Nakai & Park, 2002). Aquaculture products are highly susceptible to bacterial infections due to non-hygienic and often stressful conditions of cultivation (Muroga, 2001; Sapkota *et al.*, 2008). Susceptibility to bacterial pathogens can be partially reduced by some preventive measures: (1) avoiding pathogen transmission among culture batches; (2) improving water quality control; (3) minimizing temperature and salinity alterations; (4) reducing stress by controlling organism densities, handling and nutrition and lastly (5) the introduction of hygiene practices such as the disinfection of tanks, water and eggs (Howgate *et al.*, 1997; Nakai *et al.*, 1999; Jorquera *et al.*, 2002; Defoirdt *et al.*, 2004). Even with preventive measures, chemical anti-infective agents (antiseptics and anesthetics) are still used to reduce susceptibility to bacterial pathogens (Fauconneau, 2002; Defoirdt *et al.*, 2004; Sapkota *et al.*, 2008). Antibiotics are commonly used worldwide for therapeutic purposes (Fauconneau, 2002; Sapkota *et al.*, 2008). However, there is a lack of regulatory legislation in the licensing of antibiotics and a need for international harmonization of those available for aquaculture use, which only exist in a limited number (Tan *et al.*, 2000; Fauconneau, 2002; Taylor *et al.*, 2002; Morrison & Rainnie, 2004; Alanis, 2005; Sandeep, 2006; Sapkota *et al.*, 2008; Almeida *et al.*, 2009; Daniel, 2009). Chemotherapy is not always successful since some antibiotics are administered primarily in food mixtures, and diseased animals show poor feeding habits (Wu & Chao, 1982; Morrison & Rainnie, 2004). Additionally, environmental and public health problems arise from the use of antibiotics: (1) selection of bacterial resistance to antibiotics due to excessive prophylactic and therapeutic use, especially in sublethal doses (Cabello, 2006); (2) the up-contaminations in the food chain related to the transference of resistant organisms between animals and humans, and among animals (Inglis *et al.*, 1991; Inglis *et al.*, 1993a; Inglis *et al.*, 1993b; Perreten, 2005); (3) microorganism substitution (Park *et al.*, 2000) and (4) the toxicity of most antibiotics and their residues to aquatic organisms and surrounding environment (Cabello, 2006). These drawbacks had led to an increased urgency in finding alternative ways to control microbial outbreaks in aquaculture (Nakai & Park, 2002; Defoirdt *et al.*, 2004; Perreten, 2005; Sandeep, 2006; Sapkota *et al.*, 2008). Several antimicrobial approaches have been proposed for application in aquaculture: disruption of bacterial quorum sensing (Defoirdt *et al.*, 2004; Defoirdt *et al.*,

2007; Bai *et al.*, 2008; Jiang & Su, 2009), probiotics (Gibson *et al.*, 1998; Moriarty, 1998; Verschuere *et al.*, 2000; Nikoskelainen *et al.*, 2003; Farzanfar, 2006), microbial matured water (Munro *et al.*, 1994; Skjermo *et al.*, 1997; Skjermo & Vadstein, 1999), antibacterial photodynamic therapy with cationic phthalocyanines and porphyrins (Magaraggia *et al.*, 2006; Almeida *et al.*, 2009), green water systems (Tendencia & de la Peña, 2003; Defoirdt *et al.*, 2007), immunostimulants (Vadstein, 1997; Bricknell & Dalmo, 2005), bacteriocins (Riley & Wertz, 2002; Shehane & Sizemore, 2002; Schöbitz *et al.*, 2006), polyculture (Tendencia, 2007) among others (Defoirdt *et al.*, 2011). The presented work will emphasize the concept of phage therapy to treat bacterial infections in aquaculture systems. Evidence in support of the effectiveness of phage therapy against bacterial infectious diseases associated to diverse areas has been accumulated and is already being applied (Inal, 2003; Housby & Mann, 2009).

3. IMPORTANT CONSIDERATIONS FOR BACTERIOPHAGE USE IN AQUACULTURE

The establishment of the causative agent of the disease is the major step in bacteriophage therapy. Then, it is necessary to select the bacteriophages that can effectively infect the target bacteria and to evaluate the potential of those bacteriophages to control bacterial diseases in aquaculture. The procedure involves several sequential steps: (1) isolation of lytic bacteriophages from the fish surroundings, using an enrichment method; (2) culture of the bacteriophages; (3) phenotypic and genotypic characterization of the bacteriophages; (4) bacteriophage typing of the target bacterium and (5) selection of lytic bacteriophages for therapeutic use. Additional work is needed to establish practical procedures for bacteriophages application in aquaculture: (6) assessment of therapeutic efficacy of the phages against experimental infections (in a laboratory) and natural infections (in field trials) and (7) recognition of the existence of virulence genes or other toxic factors in the phage. Finally, phage therapy must be established at a large scale with culture and long-term preservation methods of the phages for commercial development (Nakai, 2010).

The use of bacteriophages as bacterial control agents in aquaculture has advantages and drawbacks (Table 1), and some of the critical points are the preparation of phage stocks, the effect in field conditions, the procedure for phage administration, the effect against the natural bacterial community, the potential risk for the transference of genes related to

Table 1 Pros and cons of bacteriophage alternative.

Issue	Advantage	Disadvantage	Reference
Abundance	Ubiquous providing a large, naturally available pool of bacteriophages	Strongly lytic phage strains must be selected from the available pool	(Morrison & Rainnie, 2004)
Multiplication and self limitation	Rapid exponential replication and declining, along with bacterial growth not posing an ecological risk Repeated administration is not necessary	Makes it difficult to extrapolate from *in vitro* phage growth data to *in vivo* expectations, to interpret *in vivo* data and to generalize from one *in vivo* situation to another	(Inal, 2003; Weld *et al.*, 2004)
Host specificity	Relatively narrow host range allowing that no other bacteria will be inactivated, namely useful bacteria and the normal intestinal microflora Reduced possibility of secondary infections development and side effects are less likely to occur	The exact host bacterium causing the infection needs to be identified Strain-specific rather than species-specific, increasing the difficulty when preparing phages for highly diverse bacterial variants	(Barrow & Soothill, 1997; Carlton, 1999; Nakai & Park, 2002; Mathur *et al.*, 2003)
Isolation, selection and maintenance	The selection of strictly lytic phages, the sequencing of the hereditary material of phages and toxicity tests can minimize the risk of transference of toxic genes between bacteria	High diversity within populations of both phages and bacteria implying an understanding of their heterogeneity and ecology Needs expertise and an established set up	(Yuksel *et al.*, 2001; Wagner & Waldor, 2002; Mathur *et al.*, 2003; Brüsso *et al.*, 2004; Flegel *et al.*, 2005; Scott *et al.*, 2007; Stenholm *et al.*, 2008; Nakai, 2010)
Bacterial debris	Removal can be readily achieved by current technology	Might cause therapy to fail since it might be fatal for the organism injected	(Carlton, 1999; Inal, 2003; Morrison & Rainnie, 2004)
Administration	Through feed impregnated, injection or by immersion allowing treatment of animals at various stages from eggs to broodstock	Poor feeding habits of diseased animals Injections might be unpractical to treat a large number of animals	(Barrow & Soothill, 1997; Nakai & Park, 2002; Inal, 2003; Mathur *et al.*, 2003; Nakai, 2010)

Table 1 Pros and cons of bacteriophage alternative – continued.

Issue	Advantage	Disadvantage	Reference
Dose	Determination of precise initial dose may not be essential since page titers may increase along with bacterial infection	Limited data available on effective phages doses	(Inal, 2003; Mathur *et al.*, 2003; Nakai, 2010)
Fate	Phages decrease after killing the target bacteria being finally excreted and not posing any environmental risk	Dose of administration must account for those phages that are quickly excreted	(Barrow & Soothill, 1997; Lorch, 1999; Phumkhachorn & Rattanachaikunsopon, 2010)
Multiple infections	A mixture of phages bringing synergistic effects can be applied	All the infecting bacteria must be exactly recognized	(Carlton, 1999)
Bacteria resistance	Overcoming resistance is not difficult attending to the worldwide abundance and to the rapid mutation of phages Phage resistant colonies are not necessarily still pathogenic as selection for resistance could select against virulence	Phage-resistant mutants are fairly common and with rapid appearance Newly isolated phage requires efficiency tests	(Levin & Bull, 2004; Merril *et al.*, 2006; Sandeep, 2006; Nakai, 2010)
Immunology	A higher dose of phage can compensate for those that are rendered non-viable by interaction with neutralizing antibodies	Phage-neutralizing antibodies might prevent some proportion of the administered dose of phages from being able to adhere to the bacterial target	(Carlton, 1999; Morrison & Rainnie, 2004)
Timing of treatment	The production of phage-neutralizing antibodies is slower than the kinetics of phage action	Chronic treatments may fail due to phage-neutralizing antibodies	(Sulakvelidze & Morris, 2001; Inal, 2003)
Economic costs	Compensated by the therapy efficiency in treating diseases and might be less expensive than that of using antibiotics	Procedures required after phage therapy could become too expensive Additional costs due to the need of further research in the field	(Carlton, 1999; Matsuzaki *et al.*, 2005; Miedzybrodzki *et al.*, 2007)

virulence, the development of bacterial resistance to phage infection and aspects related to legal regulation and public opinion.

3.1. PHAGES PREPARATIONS TO BE USED IN AQUACULTURE

High purity of the lytic phages isolates and of the phage stocks is required for phage therapy. The removal of bacterial debris (as endotoxins and lipopolysaccharides) can be easily achieved (Carlton, 1999; Inal, 2003; Morrison & Rainnie, 2004) eliminating further problems (Efrony *et al.*, 2007; Phumkhachorn & Rattanachaikunsopon, 2010). Contaminated phage suspensions might be fatal for the organism injected (Morrison & Rainnie, 2004). However, this is of less concern in the treatment of most bacterial pathogens of aquatic species if phage suspensions are orally administrated (Morrison & Rainnie, 2004).

Characterization of the bacteriophages and bacteriophage typing are necessary before the application of phages to control a particular bacterial pathogen due to the high degree of phenotypic and genotypic diversity within populations of both phages and bacteria (Stenholm *et al.*, 2008). The preparation of lytic phages to control highly diverse bacterial strains is critical because phages are usually strain specific. This leads to the need of phage cocktails combining different phages. For instance, *Pseudomonas plecoglossida* is a serologically uniform bacterium and is infected by a single phage type. For other bacteria, such as *Lactococcus garvieae*, a major phage type of broad infectivity exists which can lyse more than 90% of strains isolated from fish. If a given target bacterium is highly variable in its phage types or very changeable in its phage sensitivity, large collections of therapeutic phages with different lytic activities are undoubtedly required in phage therapy practices (Nakai, 2010).

3.2. EFFICIENCY IN AQUACULTURE CONDITIONS

Assessing the efficacy of the lytic phages against experimental and natural infections is crucial before phage therapy because bacteriophage with a lytic lifecycle within a well-defined *in vitro* environment may not remain lytic *in vivo* (Sandeep, 2006). Indeed, the low *in vivo* activity of phages has been pointed as the reason why little value was attributed to

phages in controlling bacterial infections in man and animals (Nakai *et al.*, 1999; Park *et al.*, 2000). The trials for evaluation of the efficiency of the phages in field conditions also serve the purpose of establishing the dose and route of phage administration. Data available on effective phage doses are limited and mainly related to treatment of fish. However, in contrast to chemicals and other substances, determination of precise initial dose given to individual fish may not be essential in the aquaculture setting, because of the self perpetuating nature of the phages causing phage titers to increase along with bacteria in infected individuals or in pathogen-contaminated water (Nakai, 2010).

3.3. ADMINISTRATION ROUTES

The administration of phages can be done by impregnated feed or injection (Nakai & Park, 2002). Even considering that diseased fish may not feed well, the administration of phages by impregnated feed enables the treatment of a large number of fish specimens. This technique can be advantageous for bacterial infections that occur through an oral route since the intestine is also a main way for the pathogen to enter the organism, and normal intestinal flora might be unaffected, but the target bacteria will be (Nakai, 2010). Although an accurate inoculation can also be achieved by injection, this may be laborious when a large number of animals or very small animals require treatment. Nevertheless, this approach is used for a number of vaccines available in the market (Nakai, 2010). The addition of therapeutic phages in the water medium is also possible because they are likely to remain effective and stable as if they were in a liquid culture or the medium of their origin (Nakai *et al.*, 1999). This type of administration has the advantage of continuous and intimate physiological contact between the organism infected and the bacteriophage (Summers, 2001; Inal, 2003). For this reason, the immersion in phage suspensions will be more effective for organisms in which infection is initiated by bacterial colonization of the skin and gills (Nakai & Park, 2002). Furthermore, in the comparison of results between the laboratory and the field, the immersion approach allows a higher similarity between environmental and laboratory conditions because phage-bacterium interaction occurs in suspension (Summers, 2001). Bathing or immersion techniques have been valuable to treat larvae, juveniles or eggs in hatcheries as shown in the biocontrol of *Vibrio harveyi* in *Penaeus monodon* larvae (Vinod *et al.*, 2006; Karunasagar *et al.*, 2007). The use of

multiple routes of phage administration is very advantageous to aquaculture since microbial infections can occur at various stages from eggs to broodstock (Nakai, 2010).

3.4. IMMUNITARY RESPONSES

Phages are recognized by the immune system of animals as external entities and an immunologic response might develop (Pirisi, 2000; Sulakvelidze & Morris, 2001). Phage-neutralizing antibodies may decrease phage effectiveness *in vivo* (Pirisi, 2000; Sulakvelidze & Morris, 2001) and a higher dose of phage might be needed to compensate for those phages that are rendered non-viable by interaction with neutralizing antibodies (Carlton, 1999; Morrison & Rainnie, 2004). Therefore, in treating chronic or recurrent infections, it appears that one type of phage or mixtures of phages can only be used once for intravenous treatment because of prior exposure (Inal, 2003). In order to overcome immune responses, it is important to understand whether phage-neutralizing antibodies are produced and for how long they remain in circulation, which factors of the immune response of the vertebrate host are able to inactivate the phages and lysis and if phage inoculations given too early could be less effective or fail completely (Barrow & Soothill, 1997; Yuksel *et al.*, 2001; Payne & Jansen, 2003). The production of phage-neutralizing antibodies after phage administration, in aquaculture, is not documented in literature. For instance, phage-neutralizing antibodies were not detected in yellowtail that repeatedly (successive 7 days) received phage-impregnated feed or in ayu after receiving intramuscular injections of phages (Nakai *et al.*, 1999; Park & Nakai, 2003). Immune response does not pose a problem for phage therapy in invertebrates, and this approach has been proven efficient in these organisms (Efrony *et al.*, 2007).

3.5. TRANSFERENCE OF VIRULENCE GENES

The presence of virulence genes or other potentially harmful elements by phages must be assessed prior to phage therapy application. Phages may act as vectors for the transference of virulence or resistance genes transforming non-pathogenic bacteria in pathogenic strains (Wagner & Waldor, 2002; Scott *et al.*, 2007). It has been estimated that the global rate of phage-mediated genetic modification in bacteria as being up to 20×10^{15} gene transfers

events per second (Brüsso *et al.*, 2004). Munro and colleagues demonstrated that the presence of the bacteriophage *V. harveyi* myovirus-like (VHML) could confer virulence to *V. harveyi* strains explaining the large variation in pathogenicity among strains of *V. harveyi*, the causative agent of luminous vibriosis in larval prawns systems (Munro *et al.*, 2003). Two apparently unrelated bacteriophages (one from the family *Myoviridae* and the other from the family *Siphoviridae*) were found to induce increased virulence in *V. harveyi* (Flegel *et al.*, 2005). Bacteriophages may sometimes mediate the toxicity of *V. harveyi* in *Penaeus monodon* by the transfer of a toxin-encoding gene or a regulator gene controlling toxin production (Lila *et al.*, 1999). The possibility of rapid horizontal transfer of virulence factors among a bacterial population upon accidental introduction of a lysogenic phage was pointed as an inherent risk for shrimp farmers (Flegel *et al.*, 2005). Also, public health concerns related to the spread of antibiotic resistance genes arise. For example, *V. harveyi* Siphophage 1 (VHS1) loses the ability to lyse cells but retains its ability to lysogenize after boiling for 10 min, which means that cooking crustaceans may not be sufficient to fully inactivate bacteriophages that might be present in this seafood (Flegel *et al.*, 2005).

3.6. DEVELOPMENT OF BACTERIAL RESISTANCE TO PHAGE INFECTION

Altered phenotypes resulting from mutations include bacterial resistance to phage infection (Scott *et al.*, 2007). The susceptibility or resistance of bacterial strains to phage lysis is partially due to the variation of receptor molecules or modification of the host restriction system (Shivu *et al.*, 2007). The receptors to which phages are targeted on the bacterial cell surface act as virulence factors so, when bacteria develop resistance to phage, they are usually changed which results in an attenuation of virulence (Barrow & Soothill, 1997; Skurnik & Strauch, 2006). These phenotype adaptations may be attributed to a change in selective pressure on the bacterium, imposed namely by phages. Phage-resistant colonies are not necessarily as pathogenic as wild types because the selection for resistance may be accompanied by a decrease of virulence (Merril *et al.*, 2006). In chemotherapy, bacteria that develop resistance to antibiotics remain pathogenic, while in phage therapy virulence might be attenuated and phage-resistant bacteria might be less pathogenic. Therefore, the success of phage therapy might also rely on changes in bacteria-phage interaction at the level of the attachment sites (Wagner & Waldor, 2002). Research in aquaculture has

showed different results with respect to the emergence of phage-resistant bacteria. Although some reports showed no evidence of phage-resistant bacteria as a consequence of phage therapy in diseased fish or apparently healthy fish (Park & Nakai, 2003), others demonstrated this effect. The emergence of mutants resistant to phage infections was demonstrated for *A. salmonicida* although these bacteria could initially be infected by more than one phage (Imbeault *et al.*, 2006). Those mutants that were resistant to one phage were sensitive to a different phage or even more than one phage types. Resistant bacteria had a slower generation time than the original strain and a very low success of replating in tryptic soy agar. All mutants were sensitive to three or more phages, and 25% of the mutants seemed to revert to the original-strain phenotype after a first replating (Imbeault *et al.*, 2006). Phage-resistant bacteria were also detected in the treatment of beta-hemolytic streptococcicosis in Japanese flounder *Paralichthys olivaceus* (Matsuoka *et al.*, 2007). Phage-resistant *Streptococcus iniae* remained pathogenic to fish and was isolated from dead fish in the phage-treated group (Matsuoka *et al.*, 2007). These results bring into question the application of bacteriophages to control furunculosis and streptococcosis.

Multiple drug resistance can emerge as a consequence of phage administration namely by transference of resistance plasmids between bacteria of diverse origins in natural microenvironments (Kruse & Sørum, 1994). Newly transformed strains may be more or less susceptible to phages, and there is still the need to find a phage specific for the newly emerged strain. However, phages are abundant and widely distributed in nature and also undergo mutations, some of which can match bacterial genetic changes, restoring affinity (Carlton, 1999; Sulakvelidze *et al.*, 2001; Sulakvelidze & Morris, 2001; Kutter & Sulakvelidze, 2005). The multiple tactics of phages to avoid, circumvent or subvert antiviral mechanisms of bacteria were revised by Labrie and co-authors (Labrie *et al.*, 2010). Bacteria that become resistant to a certain type of phage can still be infected by other phage types that can be isolated from nature (Carlton, 1999). The use of formulated mixtures of phages (phage cocktail), phage lysins alone, or the combined use of a phage cocktail and antibiotics are attractive strategies because the efficiency of treatment may increase and the emergence of resistant mutants minimized (Thiel, 2004; Petty *et al.*, 2007). However, it has been reported that adjuvant use of an antibiotic could sometimes decrease the efficiency of phage therapy, and combined treatments may enhance the problem of resistance (Mathur *et al.*, 2003; Payne & Jansen, 2003).

Some of the measurements to control the emergence of phage-resistant mutants involve: (1) using mutant phage derived from the phage that was initially active against the wild-type bacteria (Carlton, 1999; Almeida *et al.*, 2009; Labrie *et al.*, 2010); (2) using a newly isolated phage; (3) using a mixture of different strains of phages that would prevent the emergence of a resistant bacteria during the treatment (Nakai & Park, 2002; Thiel, 2004; Petty *et al.*, 2007); (4) using different therapeutic strategies in combination or (5) using lysins rather than bacteriophages (Inal, 2003; Matsuzaki *et al.*, 2005). The problem of resistant bacteria is critical and needs further research.

4. LARGE-SCALE APPLICATION OF PHAGE THERAPY: REGULATORY AUTHORITIES, PUBLIC AND SCIENTIFIC AWARENESS

The use of phages at the production scale is promising either for obtaining products with reduced bacterial loads or to inactivate microorganisms in the surroundings improving sanitary conditions (Inal, 2003). Additionally, the prophylactic use of a phage cocktail could improve the water quality of aquaculture plants by reducing the concentration of the bacteria most common in aquaculture animals (Withey *et al.*, 2005). This is of particular importance considering that bacteria resistant to antimicrobials might be present in microenvironments such as water, pelletized feed (Miranda & Zemelman, 2002) and bed sediments (Kerry *et al.*, 1994). However, at present, phage-based therapeutic items have not yet been produced and/or approved for aquaculture use.

Approvals from the United States Department of Agriculture (USDA) and Food and Drug Administration (FDA) (21CFR172.785) were given to the company Intralytix for the use of bacteriophage-based preparations as food additives in ready-to-eat foods. The advantage of this phage additive is that it does not add flavor, aroma or nutritional value to food and is viewed as being a natural and a preservative-free alternative. Approvals were also given by the Environmental Protection Agency (EPA) authorizing the use of a mixture of phages on food-handling surfaces (EPA registration 74234-1.). In 2007, the USDA also approved an OmniLytics bacteriophage product designed to be sprayed, misted or used as washing solution on cattle, in order to reduce the concentration of *Escherichia coli.* These approvals illustrate the level of concern of the regulatory authorities in relation to the microbial threat

and the need for consumers to have an alternative way of assessing microbiologically safe products.

Some reluctance in the use of live viruses can be overcome by diffusing the knowledge that phages are ubiquitous, already occurring naturally in all the places bacteria are found, namely in foods (Inal, 2003). Scepticism shared by many scientists regarding the potential of phage therapy in the treatment of bacterial diseases was based on the poor quality of the science behind the early clinical trials (Sulakvelidze & Morris, 2001). The sources and the methods of production of the bacteriophage suspensions, the characteristics of the phage formulations such as the volume to be administered and the concentration of the phage particles along with the frequency of administration were not documented well enough (Sulakvelidze & Morris, 2001; Morrison & Rainnie, 2004). Recent successes of phage therapy experiments conducted in controlled conditions provided strong scientific evidence in favor of bacteriophage therapy. Rigorous standardization of experimental designs throughout documentation and rigorous control must be assured in all studies (Morrison & Rainnie, 2004).

The actual microbial threat in aquaculture and the need to fulfilling demands of consumer will ultimately conduct to the approval of more bacteriophage-based antimicrobials.

5. MILESTONES OF PHAGE THERAPY IN AQUACULTURE

Phage applications began in medicine, agriculture, food industry and waste water treatment. Phage therapy and prophylaxis have already been tested in a variety of species, including mice, rabbits and sheep. In aquaculture, phages are only now being more broadly explored, and the concept of phage therapy is extending to diverse aquatic organisms, in addition to fish, whether or not they originate from aquaculture.

Some of the achievements related to bacteriophage therapy in aquaculture, including phage isolation and potential applications, are summarized in Table 2. This compilation of information contributes to the identification of some of the most relevant aspects of the use of phages as antimicrobial agents in aquaculture while helping to understand the evolution of phage therapy in aquaculture.

Table 2 Research on bacteriophages potential in aquaculture.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Japanese eel *Anguilla Japonica*	*Edwardsiella tarda*	Edwardsiellosis	Phages ET-1	Pond water in Taiwan	*In vitro* experiment	Mortality (92.6%) affected 25 of 27 *E. tarda* strains and reduced the bacterial count to less than 0.15% when the bacterial suspension of 1.2×10^{12} cells ml^{-1} was infected with phages ET-1	(Wu & Chao, 1982; Yamamoto & Maegawa, 2008)
Yellowtail *Seriola quinqueradiata*	*Lactococcus garvieae* formerly *Enterococcus seriolicida* (Eldar *et al.*, 1996)	Lactococcosis	*Siphoviridae* (PLgY)	Cultures of *L. garvieae* isolated from diseased yellowtail		Of 26 strains of *L. garvieae* examined, 24 were sensitive to the phage but 2 strains of *L. garvieae* and another 22 species including fish- and shellfish-pathogenic bacteria (used to determine the host range of the phage) were not	(Park *et al.*, 1997; Park *et al.*, 1998)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Yellowtail *Seriola quinqueradiata*	*Lactococcus garvieae* formerly *Enterococcus seriolicida* (Eldar *et al.*, 1996)	Lactococcosis	*Siphoviridae* (PLgY-16, PLgY-30 and PLgW-1)	PLgW-1: natural seawater PLgY-16 and PLgY-30: *L. garvieae* culture isolated from diseased yellowtail	*In vitro* survival: phages inoculated in the test media (10^4 to 10^5pfu $ml^{-1}g^{-1}$) *In vivo* survival and phage treatment experiment: administration of PLgY-16 intraperitoneally ($10^{7.5}$ pfu $fish^{-1}$ and $10^{7.2}$ pfu $fish^{-1}$) and orally ($10^{7.4}$ pfu g^{-1} and $10^{7.9}$ pfu g^{-1})	Anti-*L. garvieae* phages persisted well in various physicochemical and biological conditions, except for low acidity. The survival rate was much higher in yellowtail that received intraperitoneal injection of the phage after challenged with *L. garvieae* and higher protective effects were achieved by injections administered at the earlier time. Both intraperitoneally and orally administered phage had a protective effect on experimental *L. garvieae* infection	(Nakai *et al.*, 1999)
Ayu fish *Plecoglossus altivelis*	*Pseudomonas plecoglossicida*	Bacterial hemorrhagic ascites disease	*Myoviridae* (PPpW-3) and *Podoviridae* (PPpW-4)	Diseased ayu and water from fish farms	Oral administration of phage-impregnated feed (10^7 pfu g^{-1})	Protective effect against experimentally induced infection Inhibition of bacterial growth in water (prophylactic use of phages to prevent horizontal transmission of the pathogen)	(Park *et al.*, 2000; Nakai & Park, 2002)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Japanese eel *Anguilla japonica*	*Aeromonas hydrophila* and *Edwardsiella tarda*	Hemorrhagic septicaemia and edwardsiellosis	Several bacteriophages of *A. hydrophila* and *E. tarda*	Pond water samples of Southern Taiwan	Pure culture of host-phages Pond water experiment: *A. hydrophila* (6×10^5 cfu ml^{-1}) prior to phage introduction in the water	In pure culture, the host concentration was reduced 3 orders of magnitude in 2 h when the multiplicity of infection (moi) was above 11.5 at 25ºC. In the pond water the number of *A. hydrophila* dropped 250 folds at phage moi of 0.23 in 8 h with simultaneous phage multiplication to 10^6 pfu ml^{-1} in the water. The surviving hosts (85%) were still vulnerable to the phage. *E. tarda* dropped rapidly even in the absence of phage in the pond water after 48 h	(Hsu *et al.*, 2000)
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	Luminous vibriosis	*Myoviridae* (VHLM)	Extracted from a toxin-producing strain of *V. harveyi* isolated from moribund prawn larvae		VHML showed a narrow host range and an apparent preference for *V. harveyi* rather than other 63 *Vibrio* isolates and 10 other genera	(Oakey & Owens, 2000; Oakey *et al.*, 2002)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Ayu fish *Plecoglossus altivelis*	*Pseudomonas plecoglossicida* PTH-9802 strain	Bacterial hemorrhagic ascites disease	*Podoviridae (*PPpW-3), *Myoviridae (*PPpW-4) and a mixture (PPpW-3/W-4)	Diseased ayu and pond water	*In vitro* and *in vivo* experiments, the last using laboratory tanks and a commercial fish culture pond. Oral administration of phage-impregnated feed (10^7 pfu $fish^{-1}$)	*In vitro* study showed that PPpW-4 inhibited the growth of *P. plecoglossicida* and a mixture of the phages induced the highest inhibitory activity. High protection against water-borne infection was also showed. Neither phage-resistant organisms nor phage-neutralizing antibodies were detected in diseased fish or apparently healthy fish	(Park & Nakai, 2003)
Brook trout *Oncorhynchus fontinalis* formerly, *Salvelinus fontinalis*	*Aeromonas salmonicida* HER 1107	Furunculosis	Bacteriophage HER 110		Addition in the open water of the aquarium of bacteriophage suspensions (10^9 pfu ml^{-1})	Delayed of the infection (7 days) More than one phage could infect the bacteria strain and resistant mutants to phage HER 110 were sensitive to other phages, had a slower generation time, and very low success of replating	(Imbeault *et al.*, 2006)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	Luminous vibriosis	*Siphoviridae*	Water from shrimp farm of the West coast of India	Eighteen-day-old shrimp were challenged with the bacteria (10^5 cells ml^{-1}) Laboratory trial: (1) bacteriophage suspension (10^9 pfu ml^{-1}) added initially and after 24 h (another 0.1 ml); (2) only once initially with 0.1 ml of the phage suspension; (3) no addition. Hatchery trial (in triplicates): (1) treatment with bacteriophage (10^9 pfu ml^{-1}) at the rate of 200 ppm daily so that phage concentration in the water was 2 x 10^5 pfu ml^{-1}; (2) treatment with antibiotics (Oxytetracycline 5 ppm, Kanamycin 10 ppm daily); (3) no treatment	Laboratory trial showed that survival of *Penaeus monodon* larvae was enhanced 80% with treatment with two doses of bacteriophage as compared with the control (25%) Hatchery trial: survival in the control tank was only 17%, while in antibiotic treated tanks was 40% and in the bacteriophage treated tank was 86% This study shows that bacteriophages have excellent potential in management of luminous vibriosis in aquaculture systems	(Vinod *et al.*, 2006)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	Luminous vibriosis	Lytic bacteriophages against *Vibrio harvevi*, two from *Siphoviridae*	Three from oyster tissue and one from shrimp hatchery water	Tanks with post-larval 5 stage larvae, showing luminescence and mortality were used Bacteriophage treatment (two tanks): one suspension (2 x 10^6 pfu ml^{-1}) was added by day following the order: Viha10, Viha8, Viha10 and Viha8 Chemotherapy (two tanks): oxytetracycline (5 mg L^{-1}) and kanamycin (10 mg L^{-1})	Bacteriophage treatment resulted in over 85% survival of *Penaeus monodon* larvae The normal hatchery practice of antibiotic treatment resulted in a survival ranged from 65 to 68% This study shows that bacteriophages could be used for biocontrol of *V. harveyi*	(Karunasagar *et al.*, 2007)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Atlantic salmon *Salmo salar*	*Aeromonas salmonicida* subsp. s*almonicida* 78027	Furunculosis	Combination of bacteriophages O, R and B		Juveniles of Atlantic salmon were challenged with the bacteria by intraperitoneal injection Phage treatments: (1) intraperitoneal injection (1.9 x 10^8 pfu $fish^{-1}$); (2) oral administration (1.88 x 10^5 pfu g^{-1} $fish^{-1}$ daily for 30 days), bath (1.04 x 10^5 ml^{-1} daily for 30 days) and intraperitoneal injection (6.25 x 10^7 pfu $fish^{-1}$) Chemotherapy: oxolinic acid (10 mg kg^{-1} bw^{-1} day^{-1}) for 10 days	Fish injected with bacteriophage, immediately after challenge with the bacteria, died at a significantly slower rate than those that were either not treated with bacteriophage, or treated 24 h post-challenge but 100% mortality was observed. The effects of oral, bath and intraperitoneal injection phage treatment were compared with chemotherapy using an indirect cohabitation challenge. No protection was offered by any of the bacteriophage treatments, compared to the positive challenge group, although significant protection was offered by the oxolinic acid treatment. It was also shown that bacteriophage resistant bacteria isolates could be recovered from dead juveniles in all the treatment groups	(Verner-Jeffreys *et al.*, 2007)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Japanese flounder *Paralichthys olivaceus*	*Streptococcus iniae*	Streptococcosis		Fish culture environments	Fish were injected intraperitoneally with *S. iniae* PSi402 and 1 h later intraperitoneally injected with a mixture of two or four phage isolates	Mortality of fish receiving phages was significantly lower than those of control fish without phage treatment in all trials. However, as phage-resistant *S. iniae* were frequently isolated from dead fish in the phage-treated group, further investigations are required to establish phage therapy of the disease	(Matsuoka *et al.*, 2007)
Penaeid shrimp	*Vibrio harveyi*	Luminous vibriosis	Seven bacteriophages specific to *Vibrio harveyi* (Viha1 to Viha7), six from *Siphoviridae* and one *Myoviridae* (Viha4)	Coastal aquaculture systems like shrimp farms, hatcheries and tidal creeks along the East and West coast of India		All the phages were found to be highly lytic for *V. harveyi* and had different lytic spectrum for the large number of isolates tested Three of the phages (Viha1, Viha3 and Viha7) caused 65% of the strains to lyse while Viha2, Viha4 and Viha6 caused 40% of the host strains to lyse. Only Viha5 had a narrow spectrum (14%) Six of the seven phages isolated had a broad lytic spectrum and could be potential candidates for biocontrol of *V. harveyi* in aquaculture systems	(Shivu *et al.*, 2007)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Shrimp	*Vibrio harveyi*	Luminous vibriosis	*Siphoviridae* (VH1 to VH8)	Shrimp farm	*In vitro* experiment	All the isolates of bacteriophage (VH1-VH8) caused lysis of the host bacterial cells within 2 h. The propagation curve for each phage shows a burst time started from 1-10 h. Bacteriophages of *Vibrio* sp. might be effectively used in vivo as biological agents to control these pathogenic bacteria in aquaculture systems	(Srinivasan *et al.*, 2007)
Catfish	*Edwardsiella ictaluri*	Enteric septicemia	*Siphoviridae* (ΦeiDWF and ΦeiAU)			Initial characterization of these bacteriophages has demonstrated their potential use as biotherapeutic and diagnostic agents associated with enteric septicemia of catfish	(Walakira *et al.*, 2008)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Rainbow trout *Oncorhynchus mykiss* **and other species of trouts**	28 different strains of *Flavobacterium psychrophilum*	Rainbow trout fry syndrome (RTFS) and bacterial coldwater disease (BCWD)	Twenty-two bacteriophages (FpV-1 to FpV-22)	Water and sediment samples from Danish freshwater rainbow trout farms		The most potent phages belonged to genome size group 1 (FpV-1 to FpV-4) and group 2 (FpV-5 to FpV-11), which together infected 24 of the 28 *F. psychrophilum* strains examined, including strain 950106-1/1, which is highly pathogenic to rainbow trout. This range of host strains was mainly covered by phages FpV-5, FpV-6, pV-8, FpV-9, and FpV-11. Moreover, FpV-9 also had the highest infection efficiency of the analyzed phages, and apparently, a combination of FpV-4, FpV-9, and FpV-21 would seem to constitute the most potent cocktail of the isolated phages, together infecting 24 of the 27 Danish *F. psychrophilum* strains, with 20 of the 24 phage-host interactions being lytic	(Stenholm *et al.*, 2008)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Ayu fish *Plecoglossus altivelis altivelis*	*Flavobacterium psychrophilum*	Systemic bacterial coldwater disease (BCWD)	*Myoviridae* (PFpW-3, PFpC-Y), *Podoviridae* (PFpW-6, PFpW-7), and *Siphoviridae* (PFpW-8)	Pond water from Japanese ayu farms		PFpW-3 had high infectivity for *F. psychrophilum* isolated from ayu and other fish and proved to be efficient for the reduction of bacterial growth	(Kim *et al.*, 2010)
Shrimp	*Vibrio harveyi* CS101	Luminous vibriosis	*Siphoviridae* (Phage PW2)	Shrimp pond water		The phage adsorption rate increased rapidly in the first 15 min of infection to 80% and continued to increase to 90% within 30 min of infection. The stability of phage PW2 was dependent on temperature and pH. It was inactivated by heating at 90°C for 30 min and by treating at pH 2, 3, 11and 12. From its one-step growth curve, latent and burst periods were 30 and 120 min, respectively with a burst size of about 78 pfu per infected center. Six structural proteins were detected	(Phumkhachorn & Rattanachaikunsopon, 2010)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Phyllosoma larvae of the tropical rock lobster *Panulirus ornatus*	*Vibrio harveyi*	Luminous vibriosis	Six bacteriophages from Siphoviridae (VhCCS-01, VhCCS-02, VhCCS-04, VhCCS-06, VhCCS-17 and VhCCS-20) and two from *Myoviridae* (VhCCS-19 and VhCCS-21)	Water samples from discharge channels and grow-out ponds of a prawn farm	Nine cultures of *V. harveyi* strain 12 Bacteriophage treatments (in triplicate): (1) addition of phage VhCCS-06 (1 ml) 2 h after inoculation; (2) addition of phage VhCCS-06 (1 ml) 6 h after inoculation	The *Myoviridae* (VhCCS-19 and VhCCS-21) were lysogenic and appeared to induce bacteriocin production in a limited number of host bacteria (*V. harveyi* strain 12) One *Siphoviridae* phage (VhCCS-06) could delay the entry of a broth culture of *V. harveyi* strain 12 into exponential growth, but could not prevent the overall growth of the bacterial strain. This effect was most likely because of multiplication of phage-resistant cells	(Crothers-Stomps *et al.*, 2010)

Table 2 Research on bacteriophages potential in aquaculture - continued.

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Catfish *Clarias batrachus*	*Flavobacterium columnare*	Columnaris disease	Nine bacteriophages (FCP1–FCP9), FCP1 belong to *Podovariedae*	Water and bottom sediments	FCP1 phage was inoculated intramuscularly with virulent bacterial isolate (FC8) and post inoculated with FCP1phage at 10^8:10^6 :: cfu: pfu through intramuscular, immersion and oral administration	Protective effect (100% survival) After 6 h of phage treatment host bacterium concentration reduced (less than 10^{-3} cfu ml^{-1}) in the sera, gill, liver and kidney. The sera of dose 1 (4.55 x 10^6 pfu ml^{-1}) and dose 2 (9.15 x 10^6 pfu ml^{-1}) treated fishes mean log_{10} cfu value reduced by 3 logs (58.39%) and 5 logs (73.77%) at 96 h, respectively	(Prasad *et al.*, 2011)
Catfish	*Edwardsiella ictaluri*	Enteric septicemia	*Siphoviridae* (ΦeiDWF, ΦeiAU and ΦeiMSLS)	Water of aquaculture ponds		The genomic analysis revealed that these are virulent phages, lacking the capacity for lysogeny or expression of virulence genes	(Carrias *et al.*, 2011)

5.1. FISH

The most successful use of phage therapy in aquaculture, already in practice, has been demonstrated in the control of fish pathogens (Nakai & Park, 2002; Inal, 2003; Park & Nakai, 2003; Skurnik & Strauch, 2006). The applicability of phages for biological control of fish pathogens was first mentioned in 1981 and the earliest attempt to verify the therapeutic potential application of phages to aquacultured japanese eel (*Anguilla japonica*) was reported 1 year later (Wu *et al.*, 1981; Wu & Chao, 1982). The results showed that *Edwardsiella tarda* could be inactivated in the water system, allowing for an economical method of disease control (Wu & Chao, 1982). Although some other attempts were made, reliable field experiments on phage therapy in fish were developed in 1997 when the protective effect of bacteriophage on experimental *L. garvieae* infections in yellowtail (*Seriola quinqueradiata)* was observed (Nakai *et al.*, 1999). At that time, there was a commercial vaccine available against lactococcosis, but yellowtail aquaculture had no alternatives to chemotherapy and drug-resistant strains of *L. garvieae* were frequent (Nakai *et al.*, 1999). Primarily, the virulent bacteriophage specific to *L. garvieae*, designated as PLgY, was isolated from diseased fish and identified as a member of the family *Siphoviridae* (Park *et al.*, 1997). The *L. garvieae* strains were then distributed into groups according to the susceptibility to a set of phages isolated from fish and culture environments (Park *et al.*, 1998).

The application of bacteriophages in cultured ayu fish (*Plecoglossus altivelis*) was of particular importance, it enabled the control of *P. plecoglossicida* when licensed chemotherapeutic compounds were still not licensed for aquaculture and the control was only addressed by reducing the predisposing factors could be used. The most relevant findings that lead to the proposal of the use of phages for prophylactic and therapeutic purposes in fish pathology were that: (1) phage-impregnated food protected fish against experimental infection, indicating high level of phage activity *in vivo* (Park *et al.*, 2000; Park & Nakai, 2003); (2) phage suspension could be also used in water to prevent transmission of pathogens; (3) neither phage-resistant organisms nor phage-neutralizing antibodies were detected in diseased fish or apparently healthy fish and (4) phages reduced the frequency of infections outbreaks and fish mortality (Park & Nakai, 2003).

The results obtained with these fish models had served as reference for biocontrol approaches of a large number of species of fish in aquaculture thus indicating the potential of phage to control bacterial diseases of fish from aquaculture (Nakai & Park, 2002). Imbeault and colleagues suggested that bacteriophage combinations could be successfully used in preventive programs on fish farms (Imbeault *et al.*, 2006). Although prophylactic use of phages was recommended, bacteriophage therapy failed to treat furunculosis caused by *Aeromonas salmonicida* in farmed brook trout (Imbeault *et al.*, 2006) and in Atlantic salmon (Verner-Jeffreys *et al.*, 2007). Although safety problems associated with both approaches were not found, these unsuccessful trials suggested that furunculosis is not readily controllable by application of bacteriophage (Imbeault *et al.*, 2006; Verner-Jeffreys *et al.*, 2007). However, published reports on the etiologic agent of furunculosis presented the possibility of phage treatment (Ackermann *et al.*, 1985; Hidaka & Kawaguchi, 1986; Kay & Trust, 1991; Olivier, 1992; Wiklund & Dalsgaard, 1998; Roberts *et al.*, 2002). Even though these studies have provided considerable documentation of the existence and characteristics of bacteriophages of *A. salmonicida* subspecies *salmonicida,* it is unclear whether the host range of the *A. salmonicida* phages extends to any of the various atypical *A. salmonicida* subspecies (Morrison & Rainnie, 2004), and the treatment of furunculosis is still a challenge to phage therapy. Nevertheless, bacteriophage therapy was successfully used to treat columnaris disease in the catfish, *Clarias batrachusm*, caused by *Flavobacterium columnare*. Results demonstrated that the phage FCP1 exhibited broader host range to lyse 9 out of 15 isolates of *F. columnare*. After treatment gross symptoms disappear, bacteriological as well as phage detection tests were negative, and all the experimentally infected *C. batrachus* survived (Prasad *et al.*, 2011).

5.2. CRUSTACEANS

5.2.1. SHRIMPS

Antibiotics are commonly used in shrimp farming to prevent or treat disease outbreaks (Holmström *et al.*, 2003). *Vibrio harveyi* has become recognized as a serious cause of disease, particularly of marine invertebrates, namely the economically important penaeid shrimp (Karunasagar *et al.*, 2004; Austin & Zhang, 2006). Antibiotics used in the hatchery are sometimes ineffective in controlling luminous bacteria, when antibiotic-resistant *V.*

harveyi emerges in larval tanks causing serious mortality in *P. monodon* larvae (Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.*, 1994). It is estimated that many of the shrimp farmers use different antibiotics prophylactically, some on a daily basis (Holmström *et al.*, 2003). There is evidence of antibiotic resistance in shrimp aquaculture (Tendencia & de la Peña, 2001), but data on the use of phage therapy applied to invertebrates, like shrimp, are very scarce. A bacteriophage isolated from hatchery water proved to be infect *V. harveyi*, suggesting its potential as a biocontrol agent of luminous vibriosis. In laboratory microcosms, the addition of bacteriophages decreased the concentration of *V. harveyi* by about 2-3 log units (Karunasagar *et al.*, 2005). Similar results were obtained in an invertebrate hatchery infected with luminous vibriosis (Vinod *et al.*, 2006). Both reports showed that bacteriophages could be used for biocontrol of *V. harveyi* and that bacteriophage therapy was an effective alternative to antibiotics in the control of luminous vibriosis in shrimp hatchery systems (Karunasagar *et al.*, 2007). *In vitro* experiment confirmed that bacteriophages could be effectively used *in vivo* as biological agents to control *Vibrio* sp. in aquaculture systems (Srinivasan *et al.*, 2007). However, studies on the distribution of luminescent *V. harveyi* and their bacteriophages in shrimp hatchery indicated that the occurrence of luminescent bacteria, even in low concentrations during early larval stages, would lead to the development of luminous vibriosis, despite the presence of bacteriophages in the larval rearing tanks (Chrisolite *et al.*, 2008). The first report on the lytic spectrum of naturally occurring phages against a large collection of host bacteria obtained from different locations around the world identified six bacteriophages (Viha1, Viha2, Viha3, Viha4, Viha6, Viha7) with a wide spectrum of activity against *V. harveyi,* suggesting their potential as agents for biocontrol of luminous vibriosis in aquaculture environments (Shivu *et al.*, 2007). Phage PW2, a new lytic phage infecting *V. harveyi* CS101, was isolated and characterized in order to investigate its lytic property toward its host bacterium under controlled conditions in the laboratory (Phumkhachorn & Rattanachaikunsopon, 2010). Some successful experiments using bacteriophages for the biocontrol of luminous vibriosis, as well as studies that characterized bacteriophages that could be used in the control of this disease, have been reported in the literature (table 2). Future work will imply obtaining consistent results to corroborate the application of bacteriophage as an effective way to control luminous vibriosis in shrimps and extending research to other organisms to which this disease is associated with. The versatility of

phage therapy to control microbial infections that occur in different organisms (vertebrates or invertebrates), at various stages (from eggs to broodstock) as well as in laboratory, tanks or field applications, was showed in experiments made with shrimp larvae showing a promising potential for phage therapy (Vinod *et al.*, 2006).

5.2.2. LOBSTERS

Bacteriophage therapy was also proposed, among other alternatives, as a technique to control and remove the pathogenic *Vibrio* spp. from the larval cultures of the tropical rock lobster, *Panulirus ornatus* (Payne, 2007). *V. harveyi* has been found to be associated with diseases in spiny lobster (Vinod *et al.*, 2006). Crothers-Stomps and colleagues demonstrated that from eight bacteriophages (six phage belonged to the family Siphoviridae and two belong to the family Myoviridae), only one bacteriophage from the family Siphoviridae had a clear lytic activity against *V. harveyi* and no apparent transducing properties. Also, they have identified phage resistance as a major constraint to the use of phage therapy in aquaculture since bacteria were not completely eliminated (Crothers-Stomps *et al.*, 2010).

5.3. BIVALVE MOLLUSCS

It has been suggested that phage treatment could be a useful approach to control *Vibrio splendidus* infection (Sugumar *et al.*, 1998) in cultured larvae of the Pacific oyster, *Crassostrea gigas* (Park & Nakai, 2003) and more generally for treatment of bacterial infections in molluscan aquaculture production (Berthe, 2005). *V. harveyi* has been associated with diseases in pearl oysters (Pass *et al.*, 1987) but phages as an antimicrobial strategy to overcome this disease was not yet demonstrated. Reports on microbial control with phages for either these or any other bivalve species are still not available in scientific literature.

6. CHARACTERIZATION OF BACTERIOPHAGES FOR PHAGE THERAPY

Several bacteriophages related to major fish diseases had been identified and characterized (Rodgers *et al.*, 1981; Stevenson & Airdrie, 1984; Merino *et al.*, 1990; Yuksel *et al.*, 2001; Munro *et al.*, 2003) but not envisaging their application in phage therapy against important aquatic animal pathogens.

Male-specific RNA (F-RNA) bacteriophages (Doré *et al.*, 2000; Doré *et al.*, 2003), somatic coliphages (Chai *et al.*, 1994; Albert *et al.*, 1995; Legnani *et al.*, 1998; Nanni *et al.*, 2000; Miossec *et al.*, 2001) and bacteriophages infecting *Bacteroides fragilis*, have already been associated with bivalves (Hernroth *et al.*, 2002). These bacteriophages have been proposed as putative indicators of viral contamination in shellfish since more representative and accurate indicators to improve the microbial control of shellfish are essential (Beril *et al.*, 1996; Chung *et al.*, 1998; Grabow, 2001). However, their potential as therapeutic agents to control bacteria that cause infections in bivalves or that contaminates this food product is not yet explored.

Some phages with strong lytic potential against different pathogenic bacteria, which are considered to be responsible for serious economic damage in aquaculture, were isolated and characterized (Table1). The fish pathogen *Piscirickettsia salmonis* showed the presence of bacteria containing phage particles attached to the cell wall that appeared to eventually lyse the cell (Yuksel *et al.*, 2001). The characterization of those bacteriophages associated with *P. salmonis* was being done in the perspective of using those bacteriophages for the biological control of diseases of cultured fish (Yuksel *et al.*, 2001). Information on bacteriophages isolation and characterization could be valuable to the evaluation of the therapeutic success of the use of these phages individually or as a cocktail of phages, in the control of bacterial infections in aquaculture facilities (Shivu *et al.*, 2007; Stenholm *et al.*, 2008; Walakira *et al.*, 2008; Kim *et al.*, 2010; Phumkhachorn & Rattanachaikunsopon, 2010; Carrias *et al.*, 2011).

7. LYSINS: EXTENSIONS OF THE "PHAGE THERAPY" CONCEPT

Now that the mechanisms by which phages lyse bacteria are becoming elucidated, the partial use of specific phage components involved in bacterial lysis as an alternative to the whole bacteriophage particles, is being explored (Inal, 2003; O'Flaherty *et al.*, 2009). It would be easier to gain public acceptance of phage therapy by using one viral lytic enzyme rather than using a whole virus. Phage-specific lysins or phage peptides blocking cell-wall synthesis are being isolated and tested as an alternative to using whole bacteriophage particles for potential therapy (Inal, 2003). Most tailed phages encode peptidoglycan hydrolase (endolysin or lysin) involved in the release of the progeny at the final stage of lytic cycle. Lysin is able to degrade peptidoglycan directly, exerting a bacteriolytic effect within several seconds of administration. It can also destroy the cell walls of nongrowing bacteria, which are insensitive to many antibiotics (Matsuzaki *et al.*, 2005; Hermoso *et al.*, 2007). The simultaneous administration of two lysins that have different peptidoglycan cutting sites has a synergistic effect. With the exception of the lysin of an enterococcal phage, lysins are fairly specific for bacterial species as well as phages themselves, indicating that phage lysin can very likely eliminate the targeted bacteria without disturbing the normal flora (Matsuzaki *et al.*, 2005). Other advantages and drawbacks of the use of these lytic enzymes were summarized elsewhere (O'Flaherty *et al.*, 2009). Various lysins have been proven efficient in inactivating *Bacillus anthracis* (Schuch *et al.*, 2002; Yoong *et al.*, 2006), *Streptococcus pyogenes* (a group A streptococcus) (Nelson *et al.*, 2001), *Enterococcus faecalis* and *Enterococcus faecium* (Yoong *et al.*, 2004), *Staphylococcus aureus* (Rashel *et al.*, 2007) and *Streptococcus pneumoniae* (Loeffler *et al.*, 2001; Jado *et al.*, 2003; Loeffler *et al.*, 2003; Entenza *et al.*, 2005; McCullers *et al.*, 2007; Grandgirard *et al.*, 2008) both *in vitro* and in animal models naturally or artificially infected with these pathogens but research in the field of aquaculture have not yet been done.

8. FUTURE PERSPECTIVES – AN EXAMPLE

Freshwater finfish represents half of global aquaculture production (54%) being molluscs the second more produced aquaculture item in the world (24%) (FAO, 2009). Crustaceans

come next in production relevance, represented mostly by penaeid shrimps and grapsid crabs (FAO, 2006, 2009).

In mollusc and shrimp aquaculture, infectious disease is the most devastating problem (Mialhe *et al.*, 1995). Molluscan aquaculture production represents a particular situation, since very few ways to reduce disease in commercially exploited molluscs have been proposed (Berthe, 2005). The production of molluscs occurs in the natural environment using the primary productivity of the surrounding waters as food source, not requiring any food inputs for growth or implying any additional costs (Helm & Bourne, 2004; Berthe, 2005; FAO, 2006). This condition strongly limits the chemotherapeutic possibilities in an open system since it is neither practical nor safe for the surrounding environment, because of the quantity needed. Contradictory results on the use antibiotics on bivalves have been reported (Le Pennec & Prieur, 1977; Berthe, 2005; Giraud *et al.*, 2006). Furthermore, some antibiotics could affect larval development limiting both larvae and bivalve production (Nicolas *et al.*, 1996). Other alternatives to chemotherapy in bivalves have been proposed (Verschuere *et al.*, 2000; Prado *et al.*, 2010). Research into the use of phage therapy with regard to shrimp is still in its infancy (Karunasagar *et al.*, 1994; Vinod *et al.*, 2006; Karunasagar *et al.*, 2007) and the use of bacteriophages on molluscs is still not a common practice. Since bivalve molluscs are passive filter-feeders, along with the oxygen and nutrients, they accumulate various chemical and biological contaminants (Oliveira *et al.*, 2011). Autochthonous and allochthonous bacteria are a source of nutrients and a challenge to the innate immune system of bivalve molluscs (Girón-Pérez, 2010). Like bivalve molluscs, corals have no adaptative immune system and grow in open waters (coral reef). A pioneering research about the application of lytic bacteriophages of *Vibrio coralliilyticus* and *Thalosomonas loyaeana* as an alternative for preventing or treating infectious diseases of *Pocillopora damicornis* and *Favia favus* corals, respectively, demonstrated that diseases in both corals could be controlled by the respective pathogen-specific phage (Efrony *et al.*, 2007). This research was of particular relevance since it suggested phage application in an open system such as a coral reef attending that (1) the appropriate time to add the phage should be studied, (2) the development of phage resistance should be investigated and (3) the number of phages needed for a field trial should be quantified and produced. It was estimated that if 10^3 bacteriophage per ml would prevent the spread of the disease, a commercial fermentor of 100 m^3 would provide phages suspensions to treat large reef

areas of 1,000 km^2 (Efrony *et al.*, 2007). This research highlighted the potential of the use of bacteriophages in molluscan aquaculture in the view of the resemblance of these organisms in some conjectural facts.

As far as it has been studied, bacteriophages found in the environment are able to eliminate natural occurring planktonic bacteria but also pathogenic bacteria (Nakai & Park, 2002). So, the use of bacteriophages in mollusc might: (1) decrease bacterial infection and therefore economic losses and (2) decrease bacterial contamination improving the safety of mollusc products for human consumption.

Phage numbers can decline in an open system even if phage application is done with high ratios of phages to bacteria as it already has been suggested (Efrony *et al.*, 2007). Pre-harvest reduction of bacteria in bivalves from natural growing areas or produced in an open system by the use of phages might not be effective due to the natural decay of phages in the environment. Exposure to sunlight, UV radiation, desiccation, and various chemical and biological antagonists might contribute to this decline (Wommack *et al.*, 1996; Wilhelm *et al.*, 1998; Sinton *et al.*, 1999). Phage viable counts typically decline in many ecosystems, given an absence of specific host bacteria.

A variety of infectious diseases occur locally in a diversity of fish and shellfish (Nakai & Park, 2002). Therefore, for prophylactic measures to be addressed locally: (1) a rapid and well-designed diagnostic scheme for detection and identification of the existing pathogens causing diseases must be developed; (2) a detailed characterization of isolated phages must be made (their adsorption rate, lytic potential, interaction with host, among others) and (3) a well achieved knowledge of the surrounding environment, as well as the seasonal dynamics of bacterial communities, must be obtained (Mialhe *et al.*, 1995; Stenholm *et al.*, 2008; Pereira *et al.*, 2011).

The bioaccumulation of harmful microorganisms and the fact that it is traditionally consumed whole, raw or lightly cooked make shellfish a particular case for foodborne disease and a high-risk food product (Lees, 2000). Regulations for monitoring microbial quality of harvesting areas of mollusc bivalves aim to safeguard public health. Restriction to bivalve harvesting is often applied when the area does not meet the required sanitary standards (Oliveira *et al.*, 2011). The quality of harvesting areas could be indirectly improved by the use of bacteriophages in wastewater treatment plants, reducing the

anthropogenic bacterial inputs in these areas. Indeed, the use of phages for wastewater treatment processes was already suggested (Withey *et al.*, 2005).

Depuration and transposition are the only post-harvested "natural" ways of reducing the microbial loads of bivalve molluscs for human consumption (Oliveira *et al.*, 2011). Since depuration occurs in the confinement of commercial depuration tanks, phages could be added as an additional factor of improvement of the sanitary quality of bivalves and for acceleration of the depuration process, achieving safer products for human consumption. The advantage of using phage therapy in food products, which will be consumed raw or lightly cooked and for which the appearance is overvalued, relies on the fact that it will look natural as if no treatment was given: no flavor, aroma, preservatives or nutritional value will be added.

9. CONCLUSIONS

Bacteriophage therapy has been the subject of intensive research in many fields, but phage therapy against bacterial diseases in aquaculture is not yet fully investigated. Aquaculture has a great impact on the economy and involves mainly the production of different species of fish, crustaceans and molluscs. Most of the economic losses in aquaculture are associated with disease outbreaks caused by pathogenic bacteria. Bacteriophage therapy in fish pathogens from aquaculture has been already implemented. However, little information exists on phage therapy on other aquatic organisms like molluscs and crustaceans. In this work, the particular case of bivalve molluscs is discussed. Indeed, research is required in all taxonomic groups of aquaculture, particularly those to which less attention has been given, in order to minimize the impacts of disease, stimulating production and protecting public health.

Bacteriophage therapy has been suggested as an alternative for the prevention and treatment of microbial diseases in aquaculture. The use of naturally occurring bacteriophages as antimicrobial agents in aquatic environments for fish diseases and other infections showed both successful and unsuccessful results. Although phage therapy is showing to be promising, caution must be taken with the highlighted issues in this work. The impact of this biocontrol technique in the aquaculture system and on the respective

product must be taken into account. Even though it is less likely that bacteriophages pose a selective pressure for the development of resistance than conventional antibacterial compounds do, bacteria resistance should not be neglected. Both the cost of bacteriophage treatment and the delivery route should be considered. Additionally, the success of the administration of phage cocktails combined with antibiotic therapy as well as the use of lytic enzymes in aquaculture must be confirmed. Advantages and drawbacks are inherent to any antimicrobial approach, and a good management strategy would consider the use of several techniques in rotation. This could prevent and minimize the most expensive drawbacks of the actual control strategy in aquaculture. Approval by the authorities is required for the expansion of phage therapy in aquaculture. The fact that phage sprays are already approved for use in other food items shows that phage therapy is becoming a reality and that concerns related with the impacts of chemotherapy can be overcome.

The application of phages in aquaculture has some advantages mainly related to the direct attack of the infection, the versatility of administration routes, the variety of aquaculture organisms to which it can be applied (vertebrates or invertebrates, at various stages of development) as well as the possibility of use in closed or open systems. The potential for naturally occurring lytic bacteriophages to be used in therapy and prophylaxis of bacterial diseases in aquaculture is therefore wide and promising.

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General conclusions and Future work

General conclusions and Future work

Bivalves are appreciated worldwide as a food item fulfilling most of the demands of consumers (Bernardino, 2000; Fauconneau, 2002; Murchie *et al.*, 2005; FAO, 2006; Sapkota *et al.*, 2008). This is reflected on the development of economy sectors related to capture, production and trade of bivalves. The global tendency is that this sector of activity will continue to growth following the expansion of human populations (FAO, 2009).

In Portugal incomes related to the exploitation of bivalves are largely dependent on the wild capture (22.7% of the total capture of aquatic animals) although production is fairly representative in the aquaculture sector (53.5% of the total aquaculture). Harvested and produced bivalves are commercialized on national or international markets but imports largely exceed exports and the trade balance remains negative (DGPA/INE, 2011; Oliveira *et al.*, submitted-a).

Strategies towards a sustainable development of bivalve exploitation and valorization of the products in terms of food safety and economic profitability may include: (1) development of certified products and quality labels, (2) marketing strategies appealing to the benefits of seafood and its subsequent quality, (3) diversification of products, including other bivalve species and different presentations of the traditional products, (4) improving the knowledge and characterization (in a space and time scale) of favorable areas along the Portuguese coast without threatening environmental quality (Oliveira *et al.*, submitted-a)

Within the causes for the economic losses of the shellfish sector, bacterial contamination, which is shown to be chronic in shellfish growing areas, assumes relevance. Passive bioaccumulation of pathogenic bacteria by bivalves makes them a high risk product for consumers. Only bivalves meeting defined microbiological standards can therefore, be commercialized (Romalde *et al.*, 1994; Lees, 2000; Romalde *et al.*, 2002; Murchie *et al.*, 2005). On the other hand, bacterial diseases of bivalves may lead to an increased mortality within the aquaculture system and ultimately to reduced availability of bivalves for sale (Mialhe *et al.*, 1995; Berthe, 2005).

The monitoring processes underlying bivalve sector must be reappraised and improved by (1) reinforcing monitoring of shellfish growing areas, (2) considering management strategies in advance, (3) increasing the efficiency of the processes associated to the production of bivalves and (4) using alternative purification strategies (Helm & Bourne, 2004; Anrooy *et al.*, 2006).

Crucial improvements that are necessary in monitoring harvesting and production beds are: (1) the need for a better understanding of the bivalve ecology as well as of the composition of the bacterial community associated with the bivalves and with the surrounding environment, in order to apply specific measurements to that each particular area; (2) the evaluation of microbial population dynamics in space and time scale in order to identify possible trends; (3) the need for culture-independent methods to assess real bacterial community and excluding false-positives results of culture-dependent methods and (4) the need for direct detection and quantification of the pathogenic bacteria as well as characterization of the most critical bacterial community in terms of human safety (for instance creating a molecular tool box for monitoring microbial quality of bivalves), considering the lack of correlation of indicator microorganisms with other bacteria (allochthonous or autochthonous pathogenic bacteria) and viruses (Oliveira *et al.*, 2011).

The practical approach tested in this work using molecular methods represents the initial step towards the improvement needed in the monitoring of the microbiological quality of shellfish and may ultimately provide the practical basis to routine microbiological monitoring of shellfish growing areas. The fact that the method of extraction of bacterial DNA can be used in molluscs and environmental matrices of harvesting/production sites, provides a very straightforward tool for the assessment of the microbial loads in these areas as well as the basis for the decision on eventual classification of harvesting areas and depuration strategies (Oliveira *et al.*, submitted-b)

The methodology proposed might be also be used in monitoring the actual purification processes that is known to be important in improving the quality of bivalves but not fully effective in the elimination of microbial contaminants (namely autochthonous). The use of lytic bacteriophages (viruses of bacteria) or lytic enzymes was suggested as an adjuvant to the actual depuration processes and as a strategy to improve the quality of harvesting and production areas (Oliveira *et al.*, 2012). Used as therapeutic or prophylactic agents (bacteriophage therapy), phages enable a more effective and targeted elimination of the microbial loads thus decreasing economic losses and improving the safety of mollusc products for human consumption (Nakai & Park, 2002; Kim *et al.*, 2010; Nakai, 2010; Phumkhachorn & Rattanachaikunsopon, 2010; Oliveira *et al.*, 2012). This perspective will ultimately result in a safer product with added commercial value. Also, this strategy can be used as a prophylactic measurement to reduce diseases in bivalves increasing the quantity

of product available for commercial trade. Bacteriophage use as an alternative or complementary method has some good advantages mainly related to the direct attack to the bacteria, the versatility of administration routes, the variety of organisms to which they can be applied (at various stages of development) as well as the possibility to be used in both closed or opened systems (Nakai & Park, 2002; Oliveira *et al.*, 2012).

The present work intended to analyze the actual approaches associated to the monitoring of shellfish microbiological quality and to outline some alternative perspectives towards the development of this sector also contributing as a background for future research. Priorities in future investigations will be (1) creating a molecular tool box for monitoring microbial quality of bivalves, (2) evaluating dynamics of bacterial communities in a time and space scale (both in opened and closed systems), (3) the study of the vertical transference between bivalves and the surrounding environment, (4) the study of the use of bacteriophage therapy in reducing the time and improving the efficiency of the depuration process, (5) the study of the use of bacteriophage as a strategy of improving quality of the areas of harvesting and production either locally or indirectly throughout the use of bacteriophages as a tertiary treatment in wastewater treatment plants and (6) the study of alternatives to add value to bivalves (by certification and quality labeling) as well as the creation of new market opportunities by the production of new shellfish-derived products.

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Annexes

Published papers

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Review

Microbial contamination and purification of bivalve shellfish: Crucial aspects in monitoring and future perspectives – A mini-review

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ABSTRACT

Shellfish are a nutritious food source whose consumption and commercial value has risen dramatically worldwide. Although bivalve's consumption can contribute to a healthy diet, some can cause foodborne illnesses. Microbial contamination is chronic and pervasive in harvesting areas and may be passed on to the consumers. Current food safety programs intend to protect consumers. Nevertheless, bivalve's microbial contamination is underestimated and undermanaged, which can pose a potential public health risk. We intend to provide an updated overview of the microbial assessment of bivalves and emerging alternatives or complementary perspectives for the elimination of microbial contamination. Further research is needed for the improvement of public health control and the enhancement of shellfish safety.

Contents

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1. Introduction

It is believed that less processed or natural foods are healthier. Nevertheless, for some products this may be an oversimplification and represents a greater risk to consumers. Bivalve shellfish fit this description (Murchie et al., 2005). For dietetic, traditional or food availability reasons, consumption of bivalves has been rising dramatically worldwide (Fauconneau, 2002; Johnson & Hayasaka, 1988; Murchie et al., 2005). On the other hand, microbial contamination is chronic and pervasive in growing and harvesting areas. By filter-feeding from the surrounding water, bivalves bioaccumulate natural occurring or anthropogenic contaminants, arising this contamination to the consumer (Lees, 2000). Contamination includes pathogenic species capable of producing diseases outbreaks (WHO, 2010). In general, HACCP procedures and product processing applied to food products are sufficient to protect consumers from the risk of diseases. However, shellfish, because of their unique nature have their own distinct aspects of harvesting, processing and handling. Furthermore, bivalves are minimally processed, and traditionally consumed raw or lightly cooked as a whole (visceras included) (Lees, 2000; Murchie et al., 2005; Romalde et al., 1994). Recently, there has been observed an increasing concern regarding food safety, particularly in molluscan shellfish products. Extensive efforts have been pursued to assure a safe supply of bivalves, but disease and death due to viruses and naturally occurring bacteria have been observed. This might be a result of underestimated and undermanaged microbial contamination.

This mini-review focuses on critical aspects related to shellfish safety for human consumption with the aim of serving as a general reference in future investigations. The drawbacks in depuration and relaying processes, encountering potential indicators for human enteric viruses as well as indigenous marine bacteria and the methodology applied to quantify conventional indicators are pointed out. Emerging perspectives regarding the elimination of microbial contamination and the enhancement of shellfish safety are also discussed providing guidelines for future work in monitoring the health of bivalves.

2. Importance of bivalves

Bivalves, as a food component, are characteristically tender, easily digested, additive-free and minimally processed. These characteristics make them a product that almost completely fulfils the demands of consumers (Murchie et al., 2005). These animals also have high-quality animal protein content which is similar to that of milk and eggs making them a nutritive food and an important component in the human diet worldwide (Bernardino, 2000; FAO, 2006; Fauconneau, 2002; Murchie et al., 2005; Sapkota et al., 2008). This is particularly relevant in developing countries where aquatic products are often the only source of animal protein (Fauconneau, 2002).

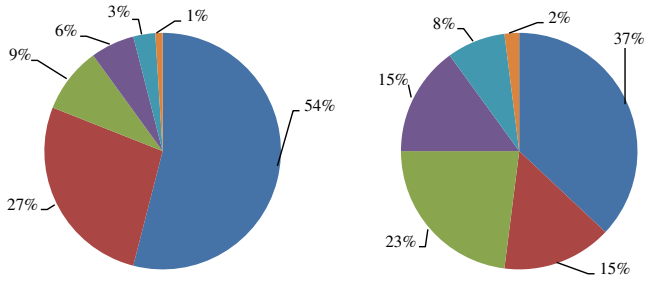

Fig. 1. World aquaculture production in quantity (left) and respective economic significance (right) of major taxonomic families groups in 2006 (FAO, 2009).

The importance of bivalve shellfish as a food supply increases if we attend to the natural resource that shellfish growing areas may represent (Johnson & Hayasaka, 1988). Dense beds of bivalve shellfish (epifaunal or infaunal species) occur in inshore estuaries with high primary productivity and have been an important source of food since prehistory (Lees, 2000). However, the aquatic environment is becoming over-exploited and as a consequence of overcatching the depletion of stocks is leading to the reduction of natural shellfish beds and to the need of human intervention in its production (Pillay & Kutty, 2005). The outcome is the development of artificial bivalve shellfish production and exploitation by the food industry (Hernroth, Conden-Hansson, Rehnstam-Holm, Girones, & Allard, 2002). Aquaculture production has been exponentially increasing and becoming one of the fastest-growing food industries, especially in Asia (Defoirdt, Boon, Bossier, & Verstraete, 2004; FAO, 2006; Sapkota et al., 2008). Fig. 1 shows aquaculture production both in quantity and in economic significance for fishes, molluscs, crustaceans and other aquatic animals in 2006 (FAO, 2009). Freshwater finfish represented half of global aquaculture production (54%) and molluscs were the second largest aquaculture product produced worldwide (24%) (FAO, 2009). The oyster culture, particularly *Crassostrea gigas*, dominates the global production of molluscs (Berthe, 2005; FAO, 2006). The Manila clam (*Ruditapes philippinarum*), the Yesso scallop (*Patinopecten yessoensis*), the blue mussel (*Mytilus edulis*) and the blood cockle (*Anadara granosa*) are also widely produced species (Berthe, 2005). Crustaceans come next in relevance, in terms of production, represented mostly by penaeid shrimps and grapsid crabs (FAO, 2006, 2009).

3. Bivalves contamination and their risk as vehicles of disease

Contamination of bivalve shellfish occurs mainly because they are suspension feeders that selectively filter small particles of phytoplankton, zooplankton, viruses, bacteria and inorganic matter from the surrounding water (Burkhardt & Calci, 2000; Defossez & Hawkins, 1997; Dunphy, Hall, Jeffs, & Wells, 2006; Lees, 2000).

For the majority of foods, proper refrigeration, storage, handling, cleaning and cooking procedures helps the consumer to control microbial risk and assure product safety. The hazards related to the contamination of bivalves by harmful microorganisms are due to their traditional cooking procedure which may not be enough to ensure consumer's safety. These circumstances make them an important vector of foodborne disease (Lees, 2000). The control of the disease risk associated with bivalves, thus, requires Hazard Analysis by Critical Control Point (HACCP) procedures together with water environment quality management of growing and harvesting areas and post-harvest product processing which might involve depuration and/or heat treatment where appropriate (WHO, 2010).

The true incidence of foodborne disease outbreaks is not known. Even thought there are routine surveillance systems worldwide that compiles the existing information on foodborne diseases, the collected information varies widely between diseases and between countries, not allowing for the numerical comparison of data on foodborne disease. Furthermore, a higher number of reported cases can be the result of a well performing surveillance system and not necessarily that people are more often sick from contaminated food. In addition, the reported number of cases for a country can include cases acquired domestically as well as acquired abroad after travel. No comparison between surveillance systems in term of their efficiency can therefore be made in a realistic way, and subsequently, trying to compare various countries data according

to their surveillance systems is not informative (Rocourt, Moy, Vierk, & Schlundt, 2003).

In general, countries who are members of the Organisation for Economic Co-operation and Development (OECD), meat (8.53%), poultry (4.14%), eggs and dairy products (14.62%) and seafood (6.63%) account for most of the foodborne diseases (Rocourt et al., 2003). When compared with these highly-consumed food products, seafood represents a quite alarming vehicle for foodborne diseases Shellfish are identified as one of the mediums of seafoodborne diseases. In New York, from 1980 to 1994, 339 seafood-associated outbreaks were reported, resulting in 3959 illnesses, 76 hospitalizations, and 4 deaths. Seafood-associated outbreaks accounted for 19% of all reported foodborne outbreaks and 10% of foodborne illnesses. Shellfish, the most frequently implicated seafood item, accounted for 64% of seafood outbreaks. The etiologic agent was confirmed for 654 (36%) of 1802 foodborne outbreaks and 148 (44%) of 339 seafood-associated outbreaks. Of the seafood-associated outbreaks, 14 (9%) were attributed to bacteria, 69 (47%) to viruses, and 65 (44%) to chemical agents. Three of the 4 seafood-associated deaths were caused by *Clostridium botulinum*; the remaining death was caused by *Vibrio vulnificus* (Wallace, Guzewich, Cambridge, Altekruse, & Morse, 1999). From 1993 to 1997, a total of 2751 outbreaks of foodborne disease involving 86,058 people were reported to the Centre for Disease Control (CDC), in Atlanta. The food vehicle was identified in only 1/3 of the outbreaks. Shellfish were often implicated in disease but did not, as opposed to some other foods, result in death. Since meat (66 outbreaks; 3205 cases) and poultry (52 outbreaks; 1871 cases) are food products that are consumed in a much larger amount, when compared to seafood, the number of cases related to shellfish (47 outbreaks; 1868 cases) is rather alarming (Olsen, MacKinon, Goulding, Bean, & Slutsker, 2000). When compared to fish (140 outbreaks; 696 cases), molluscan shellfish caused double the number of cases even though being responsible for a much lower number of outbreaks (Huss, Ababouch, & Gram, 2004; Olsen et al., 2000). In the majority of food outbreaks (67.8%) the disease agent was not identified. In 44.7% of the outbreaks caused by shellfish, the etiological agent was identified and viruses were the most frequent causative agent (23.4%) (Olsen et al., 2000). Between 1995 and 1996, 1919 outbreaks of infectious intestinal disease, affecting more than 40,000 people in England and Wales were reported to the PHLS Communicable Disease Surveillance Centre (CDSC). The food vehicle was identified for 301 outbreaks, 24 of which were reported to be due to shellfish, including 12 outbreaks attributed to eating oysters (Evans et al., 1998).

The risk of disease or death due to contaminated shellfish consumption is inherent to all consumers but the risk increases in those that suffer from underlying health disorders and are exposed to the consumption of raw bivalves. Among the high-risk population are individuals with immunosuppressive disorders (cancer patients, AIDS), achlorhydria and epilepsy, patients with diabetes mellitus, liver and chronic kidney disease and steroid dependent patients (for treatment of asthma). Pregnancy, age and alcohol abuse are also factors that may enhance the development of seafood diseases (Butt, Aldridge, & Sanders, 2004; Ripabelli et al., 1999).

3.1. Microbial contamination and human health

Foodborne disease is a public health problem which comprises a broad group of illnesses. Among them, gastroenteritis is the most frequent clinical syndrome which can be attributed to a wide range of microorganisms (Molnar, Wels, & Adley, 2006). Table 1 summarises some of the biological agents found in shellfish that can cause foodborne diseases. The risk of human intoxications is linked to the ingestion of bivalves contaminated with chemicals and biotoxins. On the other hand, the risk of human infections is related to the ingestion of bivalves contaminated with protozoan parasites, viruses and bacteria.

Chemical hazards (heavy metals, pesticides and drug-residues) are usually associated with aquaculture products or with bivalves caught from polluted waters but, in general, are uncommon in commercially harvested shellfish (Huss, Reilly, & Karim Ben Embarek, 2000; Richards, 1988).

Biotoxins, produced by dinoflagellates and diatoms (domoic acid), on the other hand are a serious health problem. These toxins, usually linked to the unpredictable growth of those microalgae (microalgae blooms), are heat resistant which means that even well cooked bivalves might still present a risk to consumer's safety. Accumulation of toxic marine algae in raw or light cooked shellfish has been associated to Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Neurotoxic Shellfish Poisoning (NSP), Amnesic Shellfish Poisoning (ASP) and Azaspiracid Poisoning (AZP) occurrences (Botana, 2008; FAO, 2004; Hallegraeff, Anderson, & Cembella, 2003; Huss et al., 2000). The level at which PSP intoxications occur in humans varies considerably according to individual sensitivity and fluctuation in the method of determination. For instance, an oral consumption of 300 μg PSP toxin per person was in some cases reported as fatal, whereas others noted the absence of toxic symptoms after an oral dose of 320 μg PSP toxin per person (Botana, 2008; FAO, 2004). Shellfish containing more than 2 μg Okadaic acid/g hepatopancreas are considered unfit for human consumption and capable of causing DSP (FAO, 2004). No mortality or chronic symptoms associated with NSP were reported and treatment is primarily supportive (FAO, 2004). For ASP the amounts of domoic acid consumed, ranged from 15 to 20 mg/person for an unaffected person to 295 mg/person for a case with severe neurological symptoms (Botana, 2008; FAO, 2004). Mild symptoms were showed after consuming 60–110 mg DA (0.9–2.0 mg domoic acid/kg body weight) and most serious cases were associated with consumption of 135–295 mg of domoic acid (1.9–4.2 mg domoic acid/kg body weight) (Botana, 2008; FAO, 2004). The lowest-observed-effect-level (LOEL) for AZP was 23–86 μg per person with a mean value of 51.7 μg/person (Botana, 2008; FAO, 2004).

The actual public health threat caused by parasites via shellfish consumption is uncertain, largely because there is minimal evidence of the transmission of infection (Robertson, 2007).

Microbial contamination is chronic and pervasive in harvesting areas. Furthermore, viruses and naturally occurring bacteria are the most often cited causative agents of disease and death related to shellfish consumption (Croci, Suffredini, Cozzi, & Toti, 2002; Huss et al., 2000; Lees, 2000; Wittman & Flick, 1995).

Shellfish-derived illnesses can have natural causes due to contaminants that are available in the environment and consequently a part of the normal biota (Shumway & Rodrick, 2009), while others can be human-generated before or after shellfish harvesting. Pre-harvesting microbial contamination (occurring naturally or as a result of human activities) includes a wide variety of viruses and pathogenic bacteria (Huss et al., 2000; Lees, 2000). Regardless of the higher abundance of indigenous marine viruses, only viruses derived from anthropogenic contamination are associated with illness in seafood consumers. Noroviruses, hepatitis A viruses, enteroviruses and adenoviruses are the viruses that are more often linked to shellfish contamination (Le Guyader, Atmar, & Albert, 2007; Lees, 2000; Muniain-Mujika, Calvo, Lucena, & Girones, 2003). Shellfish may also be contaminated post-harvesting. Potential hazard due to sub-lethally injured microbiota that may recover and multiply during later storage must be considered. Contaminant agents may also be introduced through cross-contamination, recontamination or faulty handling and processing (Huss et al., 2000; Shumway & Rodrick, 2009).

Table 1
Some biological agents implicated in seafood-related illness (Adapted from Botana, 2008; Brands et al., 2005; Butt et al., 2004; FAO, 2004; Hallegraeff et al., 2003; Huss et al., 2004; Huss et al., 2000; Muniain-Mujika et al., 2003; Ripabelli et al., 1999; Robertson, 2007).

Risk	Ethiology		Incubation period	Duration of pathology	Illness, symptoms and signs
Infection	Bacteria	*Salmonella* spp.	1–3 days	4–7 days	Gastroenteritis and Enteric (typhoid) fever. Diarrhea, fever, vomiting, abdominal cramps.
		Shigella spp.	24–28 h	4–7 days	Diarrhea, fever, abdominal cramps.
		Enterotoxigenic *E. coli*	1–3 days	3–7 days	Watery diarrhea, abdominal cramps, fever, vomiting.
		Campylobacter jejuni	2–5 days	2–10 days	Diarrhea, cramps, fever, vomiting.
		Staphylococcus aureus	1–6 h	24–48 h	Nausea, vomiting, abdominal cramps, fever, vomiting.
		Listeria monocytogenes	9–48 h 2–6 weeks	Variable	Listeriosis, septicaemia, central nervous system infections (meningitis), gastroenteritis, endocarditis, arthritis, encephalitis, osteomyelitis, pulmonary infections. Fever, muscle aches, nausea, diarrhea, violent or bursting headache and convulsions.
		Vibrio vulnificus	1–7days	2–8days	Wound infections, septicaemia, gastroenteritis. Vomiting, diarrhea, abdominal pain.
		Vibrio parahaemolyticus	2–48 h	2–5days	Wound infections, septicaemia, gastroenteritis. Nausea, abdominal cramps, watery diarrhea, vomiting.
		Vibrio cholera	24–72 h	3–7days	Epidemic and non-epidemic gastroenteritis. Profuse watery diarrhea, vomiting and dehydration causing death with hours.
	Viruses	Noroviruses	24–48 h	24–60 h	Nausea, vomiting, watery large-volume diarrhea.
		Hepatitis A virus	15–50days	2 weeks to 3 months	Diarrhea, dark urine, flu-like symptoms.
		Enteroviruses	10–70 h	2–9days	Nausea, vomiting, abdominal pain, malaise, headache, fever.
		Adenoviruses	10–70 h	2–9days	
	Protozoa parasites	*Cryptosporidium* spp.	2–28days	Days to weeks	Cramping, abdominal pain, watery diarrhea, fever, vomiting.
		Giardia lamblia	1–4weeks	Weeks	Acute or chronic diarrhea, flatulence, bloating.
		Toxoplasma gondii	6–10days	Months	Assymptomatic.
Intoxication	Biotoxins	Several species of the dinoflagellates genus *Alexandrium* spp. and the freshwater cyanophyte *Aphanizomenon flos-aquae*	30 min to 3 h	Hours to 2–3 days	Paralytic Shellfish Poisoning (PSP). Diarrhea, dizziness, nausea leading to parethesias of mouth, lips, weakness, dysphasia, dysphonia, respiratory paralysis.
		Dinoflagellates *Dinophysis* spp. and *Prorocentrum* spp.	30 min to 2 h		Diarrhetic Shellfish Poisoning (DSP). Nausea, vomiting, diarrhea, abdominal pain, chills, headache, fever.
		Dinoflagellate *Gymnodinium breve* (also called *Ptychodiscus breve,* since 2000 called *Karenia brevis*)	30 min to 3 h		Neurotoxic Shellfish Poisoning (NSP). Tingling and numbness of lips, tongue, throat, dizziness, diarrhea, vomiting, nausea, chills, sweats, reversal of temperature, hypotension, arrhythmias, cramps, bronchoconstriction, paralysis, seizures and coma.
		Mainly the Diatom *Pseudo-nitzschia pungens* f. *multiseries* and other *Pseudo-nitzschia* species	24–48 h		Amnesic Shellfish Poisoning (ASP). Vomiting, diarrhea, abdominal pain, neurological problems such as, confusion, memory loss, disorientation, seizure, coma.
		Dinoflagellate *Protoperidinium crassipes*	30 min to 24 h		Azaspiracid poisoning (AZP). Nausea, vomiting, severe diarrhea, and stomach cramps.

Viruses are frequently the cause of seafood-related infections, but hospitalizations and deaths are especially and generally related with bacteria (Butt et al., 2004).

Among the indigenous microbiota of coastal environments, the family Vibrionaceae, particularly *Vibrio parahaemolyticus*, *V. vulnificus* and *Vibrio cholerae*, is targeted as a causative agent of human disease due to the consumption of shellfish (Butt et al., 2004; Hood & Ness, 1982; Normanno et al., 2006; Ripabelli et al., 1999). These natural pathogens remain viable and cultivable in water, even in the absence of organic matter (Croci et al., 2002; Marino et al., 2005; Pruzzo, Gallo, & Canesi, 2005).

Several reports of human disease caused by *Listeria* spp., namely listeriosis, were related to seafood consumption but inconsistent results were observed (probably as a consequence of distinct coast contamination or different efficiencies in the detection and quantification methods). Furthermore, the contamination source (marine environment and processing/handling) and the seasonal fluctuations of the occurrence of these bacteria were not investigated effectively (Butt et al., 2004; Monfort, Minet, Rocourt, Piclet, & Cormier, 1998; Rodas-Suárez, Flores-Pedroche, Betancourt-Rule, Quinones-Ramirez, & Vazquez-Salinas, 2006). It is worth highlighting that there is growing evidence of the emergence of multi-resistant *Listeria monocytogenes* strains, due to the constant use of antimicrobial agents, thus representing a potential threat to human health (Rodas-Suárez et al., 2006).

The presence of *Salmonella* spp. in seafood and water may cause salmonellosis, characterized by enteric (or typhoid) fever along with gastroenteritis, abdominal cramps and diarrhea (Brands et al., 2005). *Salmonella enterica* serovar *Enteritidis* and serovar *Typhimurium* are the most common salmonella that cause infection and death (Butt et al., 2004). Enterotoxigenic *Escherichia coli*, *Campylobacter jejuni* and *Staphylococcus aureus* are also among bivalve bacterial contaminants and agents responsible for human disease (Brands et al., 2005; Butt et al., 2004).

3.2. Microbial contamination sources

The microbiological safety of bivalves as well as the suitability of coastal areas for growing and harvesting shellfish is directly related to the quality of the water in which they grow (Son & Fleet, 1980). However, water quality does not necessarily reflect the sanitary quality of shellfish harvested (Burkhardt, Watkins, & Rippey, 1992).

The increase in population density has increased the vulnerability of shellfish growing areas through shellfish exposure to human and industrial contaminants (Brands et al., 2005; Lees, 2000). Sources of human and animal fecal pollution include pet and wildlife waste, rainfall events, and river flows. Uncontrolled sewage disposal or performed without previous appropriated treatment, small river outlets or diffuse land runoff of contaminants derived from agricultural activities and septic tank leakages may also produce sporadic contamination (Hernroth et al., 2002). Shellfish growing areas are usually close to wastewater discharges or in polluted estuarine systems and bivalve contamination is usually linked to the accumulation of human and animal pathogens of fecal origin. Nevertheless, in the process of filter-feeding, bivalve shellfish are likely to accumulate a diversity of microbiological contaminants (Burkhardt & Calci, 2000; Croci et al., 2002). Considering that fecal associated pathogens available in the marine environment accumulate in bivalves by filter-feeding, thus sewage contaminants may be recycled into the human community (Hernroth et al., 2002). This gains particular importance due to the fact that bivalves may have been exposed to persistent antibiotic residues and to multi-resistant pathogens as a result of an increased use of antibiotics by humans, in aquaculture and livestock. These multi-resistant pathogens may exist in the environment and may re-enter the food chain (Hektoen, Berge, Hormazabal, & Yndestad, 1995; Lees, 2000; Rodas-Suárez et al., 2006; Sapkota et al., 2008). Furthermore, a nonculturable but viable and latent bacteria species of sanitary importance may be present in water besides the existence of various processes that control the levels of microorganisms in coastal marine environments (Troussellier et al., 1998).

Allochthonous microorganism's number may be reduced in the natural environment because of physiological, hydrodynamic and biotic factors. Some of these are: pH, temperature, salinity, oxygen concentration, amount of organic matter, sunlight, water dispersion, re-suspension, sedimentation, competition of autochthonous bacterial community for nutrients and, finally, microbial predation by planktonic organisms (Ho & Tam, 2000; Hood & Ness, 1982; Troussellier et al., 1998). The same factors cannot be applied straightforward to microorganisms naturally present in water that also constitute a health problem (Croci et al., 2002; Pruzzo et al., 2005).

3.3. Factors with influence on microbial contamination of bivalves

Environmental conditions influence the occurrence of microorganisms in seawater and, consequently, their contact with shellfish. Burkhardt and his colleagues showed that temperatures outside the range of 13–22 °C and salinities greater than 25 ppt reduce the survival of *V. vulnificus* in seawater (Burkhardt, III, Watkins, & Rippey, 1992). Annual variation of water temperature and salinity influence shellfish's physiological state and capacity of siphoning and therefore affects the bivalve's ability to selectively accumulate microbial species. Kaspar and Tamplin described that the greatest accumulation of microorganisms in hard-shelled clams occurred during certain periods in the spring, at temperatures ranging from 11.5 to 21.5 °C (Kaspar & Tamplin, 1993).

Furthermore, bivalve's inter- and intra-specie variations determine the amount of water filtered, which is between twenty and one hundred liters of water a day, independently of the environmental conditions (Richards, 1988; Robertson, 2007). This means that, bivalve molluscs feeding physiology determines the accumulation of pathogenic microorganisms filtered from the overlying water (Burkhardt & Calci, 2000; Ho & Tam, 2000). These phenomena may partially explain seasonal and geographical differences in microbial content of bivalves (Hernroth et al., 2002). The availability of edible shellfish depends on the fluctuation of microorganism (type and quantity) in the marine environment as contamination results from ingestion of accessible contaminants. The ability of accumulated microorganisms to persist and multiply in bivalve tissues, despite the natural protection of the shellfish by the bactericidal activity of the haemolymph, also influences the existence of unhealthy shellfish (Johnson & Hayasaka, 1988; Power & Collins, 1990; Pruzzo et al., 2005).

4. Ensuring safe human consumption

4.1. Controlling harvesting areas

A few years ago, investment in sewage treatment processes still had many barriers to overcome. The geographical location of the shellfish industry was used as an argumentative factor to justify the difficult and expensive task in achieving and maintain high standards of water quality. Investing in adequate sewage treatment systems was considered disproportionate in terms of the value of the shellfish industry (Lees, 2000). Environmental concerns have contributed in recent years, to the increased investment in sewage infrastructure. However, important improvements are still needed, namely appropriate discharge locations for treated water, adequate arrangements for storm water storage and treatment, tertiary treatment of effluents and adequate evaluation methodologies of the effluent microbial quality (Lees, 2000). The location of pollution inputs must be previously well identified in order to assure that quality-monitoring programs take them into consideration. This may result in the expansion of sewage infrastructures even to sparsely populated areas or other areas which represent a low sewage input (Lees, 2000). Risk management strategies for shellfish harvesting areas must be improved in other to prevent shellfish contamination (Shumway & Rodrick, 2009).

4.2. Legislation for safeguarding consumers

Adequate safeguards can be useful in minimizing the probability of shellfish microbial contamination, from harvesting to consumption, and in the protection of public health. The European Directive 2006/113/CE (Anonymous, 2006) and the European Directive 2004/41/CE (Anonymous, 2004d), the US interstate agreement set out by the Food and Drug Administration (Anonymous, 1993) or the UK Advisory Committee on Microbiological Safety of Food (Anonymous, 1998) are guidelines, based on the levels of microbiological indicators for both shellfish and overlying waters. The legislation employs a classification to the seafood harvesting areas according to bacterial indicators of sanitary quality (*E. coli*), quantified through a 5-tube 3-dilution most probable number (MPN) test. This classification determines whether shellfish can be sent for direct consumption or must be treated previously to commercialization (Lees, 2000). Table 2 summarises the European standards for bivalve shellfish beads. All shellfish sent for direct human consumption without any further processing must comply with a standard of less than 230 *E. coli* in 100 g of shellfish meat in more than 90% of samples. Shellfish harvesting from polluted (category B and C) areas is allowed when shellfish undergo previous treatment, before being commercialized. Bivalve molluscs harvested from growing areas exceeding Category A standards can be placed on the market for human consumption following controlled self-purification in tanks of clean seawater (commercial depuration), prolonged relaying in clean seawater or commercial heat treatment or processing by any other acceptable method (Jones, Howell, & O'Neill, 1991; Lees, 2000; Murchie et al., 2005). Shellfish from category C areas may, if necessary, be depurated before commercialization. However, some processes may not be effective at high levels of

Table 2
European classification of bivalves growing areas according of *Escherichia coli* (Lees, 2000).

Category	MPN of *Escherichia coli* per 100 g of seafood	Treatment required
A	≤230	Direct human consumption.
B	[230; 4600]	Depuration or relaying, to meet category A.
C	[4600; 46,000]	Protracted relaying to meet category A. Relaying to meet category B and depuration.
D	>46,000	Harvesting prohibited.

contamination, so another category is defined as D. Shellfish from those harvesting areas cannot be treated by any of the procedures previously mentioned.

The final product is sealed, labelled for traceability and commercialized giving distributors and consumers the confidence of a safe certified product (Jones et al., 1991; Lees, 2000; Shumway & Rodrick, 2009).

The U.S. Food and Drug Administration control procedures similarly rely on microorganism indicators for monitoring harvest waters in order to determine approved and restricted harvest areas and the treatment requirements prior to being released for human consumption (Lees, 2000). Category A defines the cleanest growing areas from which shellfish can be harvested and these areas are classified as "approved". Bivalve growing areas that do not comply with satisfying criteria, or without classification due to the lack of sanitary surveys, cannot be harvested for human consumption and are defined as "restricted". Harvest restriction can also be employed for short periods of time as a result of predictable or sporadic pollution. Such areas are classified as "conditionally approved" or "conditionally restricted" (Lees, 2000). The frequency of sample collection is dependent on the degree of contamination of the harvesting areas (Richards, 1988).

In many countries, these standard guidelines become very important for the regulation of shellfish harvesting and routine monitoring of overlying waters (Jones et al., 1991). However, when authorized shell fishing harvesting areas decrease, non-ethical activities such as illegal harvesting from polluted and restricted areas, wet storage of harvested shellfish in polluted waters, and other violations of legislation become problematic (Jones et al., 1991).

Other important aspects, other than the classification of growing areas, must be considered in order to reduce shellfish contamination. To achieve consumer protection and to minimize the inherent risks of shellfish consumption, legislation also sets requirements for sample collection, wet storage, bivalve self-purification by depuration and/or relaying (tank construction and operation, packaging, labelling), shellfish processing, laboratory analytical methodologies and product distribution. Regulations on food hygiene (Regulation N° 852/2004/EC) and on living bivalve molluscs (Regulation N° 853/2004/EC-Annex III Section VII) are well understood. Other regulations impose microbiological criteria for foodstuffs that set acceptable microbiological limits for all foods including live bivalve shellfish (Regulation N° 2073/2005) (Anonymous, 2004a, 2004b, 2005). Regulation N° 854/204/EC establishes specific attributes on the organisation of official controls on products of animal origin intended for human consumption (Anonymous, 2004c). At all stages, starting from the moment that the shellfish is collected until its consumption, good handling practices by applying Good Manufacturing Practice (GMP), Good Hygiene Practice (GHP) and a well designed HACCP programme are needed to prevent contamination and ensure a safe product (Huss et al., 2000; Lees, 2000; Marino et al., 2005; Shumway & Rodrick, 2009). Despite all regulations and guidelines. Sagoo and his colleagues showed that, in the UK during 2003, molluscan shellfish from retail and production premises found that 4% of 682 batches were unsatisfactory due to the presence of high levels of *Escherichia coli* (3.3%; 102 to 106 cfu g^{-1}), *V. parahaemolyticus* (0.4%; 102 to 106 cfu g^{-1}), and *Staphylococcus aureus* (0.3%; >103 cfu g^{-1}) (Sagoo, Little, & Greenwood, 2007).

5. Purification methods

Sanitary regulations rely on bacterial indicators of sewage contamination to classify shellfish harvesting waters and to estimate the efficiency of purification methods (Murchie et al., 2005). These purification procedures, used to reduce anthropogenic or natural microbial contamination of bivalve molluscs, have been used since the 1920s and are now extensively used worldwide (Lees, 2000). Unhealthy harvested bivalves purge contaminants when transferred into clean natural shellfish beds (relaying) or into tanks (depuration) (Richards, 1988; Shumway & Rodrick, 2009). Depuration consists of a flow-through or recirculation system of chemically (chlorine, ozone, iodophores, and activated oxygen) or physically (UV irradiation) disinfected water to allow purification under controlled conditions (Lees, 2000; Richards, 1988; Son & Fleet, 1980). This process usually occurs in 2 days (Lees, 2000). Relaying consists of transferring contaminated harvested bivalves to cleaner areas allowing self-purification in the natural environment for longer periods, at least two months for category C shellfish, according to EU standards (Lees, 2000; Richards, 1988). Purification processes are based on the assumption that if by filtering polluted water shellfish can become contaminated, they may also purge the contaminants by filtering clean water. Thus, microbial depuration decreases the risk for potential infections due to shellfish consumption. In fact, most consumers prefer to buy depurated products, not only because they are safer in terms of contamination, but also because they are less gritty and more palatable (Richards, 1988).

5.1. Depuration – practical considerations

Depuration efficiency is primarily related to bivalve's size, siphoning activity, and physiological conditions (Jones et al., 1991; Richards, 1988).

The type and quantity of initial contamination also accounts for depuration efficiency as more contaminated bivalves require longer depuration times and different microorganisms respond differently to the purification process. Likely, seeded (laboratory-induced) and natural-contaminated bivalves present dissimilar kinetics of contaminant elimination (Son & Fleet, 1980). Artificially contaminated molluscs depurate more rapidly than environmentally contaminated ones (Croci et al., 2002; Jones et al., 1991; Richards, 1988). Different rates of elimination also occur when bivalves are contaminated with individual or several bacteria (Son & Fleet, 1980).

Temperature and salinity are two important parameters to consider in the purification process according to the type of shellfish. Variations in environmental requirements among bivalves may reflect shellfish adaptation to *in situ* conditions. Animal stress induced by differences in water temperature, from that of the *in situ* shellfish growing areas to the process water, also influence purification time and efficiency. Lowering the temperature may help in keeping bivalves alive longer and maintain lower bacterial concentrations, however, this would also extend the period of time required for effective depuration. Shellfish conditioning, that allows shellfish to acclimate to the temperature and salinity of the water, seems necessary to ensure maximum depuration (Johnson & Hayasaka, 1988; Richards, 1988). Specific studies are required to

determine optimal conditions for shellfish microbial depuration accordingly to geographical characteristics (Johnson & Hayasaka, 1988). Differences in experimental design, such as commercial or laboratory-scale depuration systems must also be considered, as the time needed for bivalve purification differ (Jones et al., 1991). Susceptibility to temperature fluctuations is less likely in thermostatically controlled systems. Also, water volume and shellfish loading rates will affect the pH and the dissolved oxygen levels in the system. The number of bivalve layers in depuration recipients can promote increases in the microbial load as result of recontamination, obstruction of water flow and restrictions of shell opening (Richards, 1988). Depuration has a great potential as a means of purging shellfish, at least partially, of microbiological contaminants. Nevertheless, more detailed studies are needed to determine the effect of physiologically parameters, such as food availability, temperature, salinity, dissolved oxygen and shellfish state. This would allow the development of an improved depuration method (Jones et al., 1991).

5.2. Relaying – drawbacks

In contrast to depuration, where bivalves can only be held for a short period of time (maximum of 48 h), in the relaying method, molluscs can be kept for longer periods (at least two months) (Lees, 2000; Richards, 1988). In fact, in controlled purification, extended periods will reduce palatability and quality of bivalves and might even, cause bivalve mortality due to the unavailability of food. This will obviously result in a negative economic impact, due to delayed marketing and commercialization (Jones et al., 1991).

Drawbacks of relaying include: lack or availability of acceptable sanitary shellfish growing waters, early harvesting from fishermen and economical considerations namely regarding ownership rights (Lees, 2000; Richards, 1988). In addition, bivalves are more susceptible to environmental disturbances that cannot be controlled such as temperature fluctuations, water movements (tides and storms) and weather (Lees, 2000; Richards, 1988; Son & Fleet, 1980). Smothering and clogging by sediments, physiological stress, shell damage and predation are very likely to occur during the relaying process (Richards, 1988). Furthermore, water quality of relaying areas is difficult to assure. The possibility of recontamination by seasonal variations of naturally occurring bacteria populations or transient pollution (due to heavy rains and associated land runoff), may contaminate acceptable relay areas, leading to an ineffective microbial reduction (Croci et al., 2002; Ho & Tam, 2000; Lees, 2000; Richards, 1988). Assessing the efficiency of the relaying process is also difficult because the indicator microorganism's levels may fluctuate erratically during the exposure period (Ho & Tam, 2000; Richards, 1988).

In summary, eating raw or lightly steamed shellfish harvested from contaminated areas, but purified in acceptable marine waters or in artificial tanks, can still cause infection and disease in a significant percentage of the exposed population (Lees, 2000; Richards, 1988).

5.3. Microorganisms indicators – important considerations

Conventional depuration can be a viable alternative for molluscs that have been exposed to polluted waters improving their quality as a food resource, especially for those that are sold alive for raw consumption – it reduces the bacteria levels present in mollusc meat without heat processing (Johnson & Hayasaka, 1988; Jones et al., 1991; Lees, 2000).

However, the efficiency of these purification practices is questionable since it is based on bacterial indicator standards to ensure shellfish safety. The use of such indicators was made necessary by the difficulty in detecting many human pathogenic bacteria and viruses. Additionally, they avoid the need to screen for individual fecal pathogens (Scott, Rose, Jenkins, Farrah, & Lukasik, 2002). Nevertheless, there is a well known lack of correlation between the presence of bacterial indicators and viral pathogens (which are tightly attached to the internal tissues) in both shellfish and harvesting waters. Dissimilar elimination rates of indicator bacteria compared to viruses and indigenous marine bacteria are also well documented (Marino et al., 2005; Murchie et al., 2005; Romalde et al., 2002; Son & Fleet, 1980). Hence, more representative and accurate indicators are sought in order to improve the microbial control of shellfish (Formiga-Cruz et al., 2003).

The occurrence of few pathogenic bacteria in shellfish does not generally represent a high risk to public health because threshold levels necessary to cause illness far exceed those present. In contrast, viruses are infectious even in very low numbers, which makes total virus depuration essential to ensure public safety (Lees, 2000; Richards, 1988). Disease outbreaks associated with the consumption of shellfish compliant with the *E. coli* standard (less than 230 *E. coli* per 100 g), particularly in relation to viral infections, continues to be reported (Doré, Henshilwood, & Lees, 2000; Lees, 2000). It seems that viruses survive longer both in the marine environment and in the digestive tracts of bivalves compared to *E. coli* (Hernroth et al., 2002). Furthermore, there are studies reporting the detection of viruses in shellfish harvested from areas considered unpolluted, and meeting the current bacteriological standards (Muniain-Mujika et al., 2003; Romalde et al., 2002). Viral pathogens include culturable and nonculturable viruses whose detection methods are complex, laborious, time-consuming and expensive. Consequently, their use in routine monitoring is limited, hindering their establishment as regulatory standards methods (Hernroth et al., 2002; Lees, 2000; Murchie et al., 2005).

5.3.1. Indicator microorganisms – alternatives

The analysis of fecal coliforms and *E. coli* has limited predictive value for viral pathogens such as, noroviruses (NV), hepatitis A viruses (HAV), enteroviruses (EV) and adenoviruses (ADV), and alternative indicators microorganisms have been proposed (Muniain-Mujika et al., 2003). Traditional depuration does not significantly reduce the levels of Male-specific RNA (F-RNA) bacteriophages, somatic coliphages, bacteriophages infecting *Bacteroides fragilis*, or the occurrence of human pathogenic viruses, although its efficiency in reducing *E. coli* levels was confirmed (Formiga-Cruz et al., 2003). Based on these findings, the phages above mentioned have been suggested as putative indicators of viral contamination (Hernroth et al., 2002). F-RNA phages, frequently found in sewage and fecal contaminated waters, are a group of single-stranded RNA viruses that belong to the family Leviviridae and their physical and genomic properties are similar to the NV and HAV (Doré et al., 2000; Doré, Mackie, & Lees, 2003). F-RNA bacteriophages are probably more representative of the pathogenic viral kinetics in shellfish than *E. coli,* either because they are more resistant to environmental stress (U.V. irradiation), or because they have longer retention time in shellfish (due to the differences in the way they are accumulated and eliminated) or even a combination of the two (Doré et al., 2003). Virus depuration is slower than indicator bacteria clearance, requiring more than 48 h and still does not always meet acceptable criteria (Lees, 2000; Richards, 1988). In fact, recent studies suggested a 5-day depuration treatment to ensure elimination of viruses in mussels (Formiga-Cruz et al., 2003). Hence, the slower elimination kinetics of F-RNA bacteriophages in relation to *E. coli,* during depuration, appears to be representative of the kinetics of elimination of human enteric viruses (Hernroth et al., 2002). These properties associated to the simplicity of enumeration, make F-RNA phage an attractive

indicator organism for viral contamination in the marine environment (Doré et al., 2003; Hernroth et al., 2002). However, some authors have presented some reservations in terms of the fact that monitoring through this indicator will increase shellfish safety (Hernroth et al., 2002; Torrado, Henshilwood, Lees, & Romalde, 2002; Vilariño, Ribao, Henshilwood, & Romalde, 2006). Indeed, F-RNA phages have demonstrated a significant relationship to the presence of human viruses in shellfish, although showing very weak predictive capability for EV, HAV and ADV and a stronger predictive capability for NV (Formiga-Cruz et al., 2003). On the other hand, the absence of F-RNA bacteriophages appears to be a reliable indicator that enteric viruses, such as NV, are likely absent (Doré et al., 2000). Similarly to *E. coli*, F-RNA bacteriophages are not human specific, and a contamination with this phage may be associated to animal feces originated by land runoff and may not imply health risk due to NV (Doré et al., 2000). Oligonucleotide probe hybridization methods for geneotyping F-RNA bacteriophages would provide the possibility to differentiate animal-associated from human-associated bacteriophage groups (Doré et al., 2000). Somatic coliphages, viruses that infect *E. coli* bacteria, are constantly present in treated or non-treated sewage, they are non-pathogenic to humans, and are more similar to enteric viruses with respect to physical characteristics, environmental resistance to inactivation in the marine environment and resistance to treatment processes than are indicator bacteria (Cole, Long, & Sobsey, 2003). However, coliphages are able to increase their initial effluent discharge number in marine environment and in shellfish. Furthermore, they are not a specific index for pollution with human enteric viruses, as they are found in both human and other animals (Legnani et al., 1998). Male-specific (F+) coliphage (group II and III) has been pointed out as providing an additional advantage in distinguishing animal and human fecal pollution (Cole et al., 2003; Scott et al., 2002). The *Bacteroides* spp. is present in high numbers in both the human and animal gut and is a major component of human feces (Scott et al., 2002). Several studies have reported that the probability of detecting viruses increases when phages of *B. fragilis* are found, particularly, *B. fragilis* RYC2056 (Muniain-Mujika et al., 2003). The detection of *B. fragilis* phage has the advantage of being highly specific. Additionally, these phages do not replicate in the environment (Scott et al., 2002). This could be a suitable group of bacteriophages to be used as an indicator of the presence of viruses in shellfish (Muniain-Mujika et al., 2003).

Some authors propose human ADV as a molecular index of viral contamination in shellfish (Hernroth et al., 2002; Muniain-Mujika et al., 2003; Pina, Puig, Lucena, Jofre, & Girones, 1998). In fact, this virus was usually detected when EV and HAV were also found (Hernroth et al., 2002). Technical simplicity related to simpler detection methodologies of DNA viruses compared to those of RNA viruses and more sensitive and specific molecular techniques, are the advantages of using human ADV as a molecular indicator of human-specific viral fecal pollution (Hernroth et al., 2002; Lees, 2000; Muniain-Mujika et al., 2003). However, epidemiological studies for EV and ADV are difficult to perform because those infected by the viruses can act as carriers without showing any symptoms. As a result, the disease may only become apparent after the infection of another individual, probably far away from the original source (Hernroth et al., 2002; Muniain-Mujika et al., 2003). However, detection of human ADV by PCR has been proposed as a molecular parameter for monitoring the presence of human viruses in the environment, more studies are required to define the relationship between the level of viral contamination in shellfish and their potential pathogenic effect after consumption (Muniain-Mujika et al., 2003). Furthermore, ADV are present in much higher numbers than HAV or NV and therefore their value as indicators are limited (Torrado et al., 2002).

It is important to notice that environmental conditions play an important role in the accessibility, accumulation and elimination of both viral contaminants and potential indicator organisms from bivalves (Hernroth et al., 2002).

Temperature and UV irradiation are some of the factors affecting the viability and stability of viral particles in seawater and virus removal during depuration (Formiga-Cruz et al., 2003; Lees, 2000). Somatic coliphages have been indicated as ensuring a better marine water quality monitoring than F-RNA phages and fecal coliforms because the formers are less susceptible to longer solar wavelengths, which are predominant in the marine environment (Sinton, Finlay, & Lynch, 1999). It was found that the probability of a positive detection of any of the pathogenic virus decreases as the temperature of shellfish growing waters increases (Muniain-Mujika et al., 2003). The levels of the potential indicators also change with temperature. The distribution of F-RNA bacteriophages has been shown to be seasonal, with higher levels during the winter; this trend was also observed in the identification of typified NV, but not for the detection of ADV, EV, or HAV (Doré et al., 2003; Formiga-Cruz et al., 2003; Hernroth et al., 2002). In fact, NV gastroenteritis has been considered a "winter vomiting disease" (Doré et al., 2000; Hernroth et al., 2002). Phages infecting *B. fragilis*, in contrast to ADV, decrease in number with temperature (Hernroth et al., 2002). The selection of an indicator microorganism is further complicated when focusing on the potential pathogenicity of some indigenous marine bacteria (Murchie et al., 2005). Autochthonous bacteria are not implicitly associated with the presence of fecal contamination. Thus classical indicators of fecal contamination do not predict their presence in shellfish or water (Hood & Ness, 1982). Furthermore, one of the basic criteria for a good indicator organism is that the indicator must survive as long as the pathogen, but *E. coli* does not survive in estuarine water as well as *V. cholera* (Hood & Ness, 1982). Several authors have confirmed the lack of correlation between traditional indicators and the presence of *Vibrio* spp. (Hood & Ness, 1982; Marino et al., 2005; Normanno et al., 2006; Ripabelli et al., 1999). Seasonal variations in the indigenous bacteria populations make it extremely difficult to select safe waters for mollusc harvesting (Croci et al., 2002). Fecal indicators provide an inadequate index of microbiological safety for naturally occurring vibrios and underestimate the efficiency of the depuration process. Like enteric viruses, *Vibrio* spp. has a different response to the depuration process from that of *E. coli*. It is possible to obtain edible shellfish from anthropogenically-contaminated shellfish, but the same measure cannot be used with shellfish contaminated by naturally occurring bacteria. Similarly, it is expected that the elimination of microorganisms derived from fecal contamination and those included in shellfish natural microflora would be different (Croci et al., 2002; Jones et al., 1991). In fact, indigenous marine bacteria do not depurate well and may even multiply in depurating shellfish tanks and pumping systems (Richards, 1988). Therefore, a more appropriate indicator must be developed to reduce seafood illness risk derived from *Vibrio* spp.. Enterococci have been proposed as a more appropriate indicator of the risk from vibrios than *E. coli* (Marino et al., 2005). It is also important to be aware of the fact that none of the current regulations include specific tests for indigenous marine bacteria (Murchie et al., 2005). Thus, the need to improve shellfish-borne disease control strategies must also focus its attention on *Vibrio* spp. (Ripabelli et al., 1999).

5.4. Methodologies for monitoring bivalve's safety – critical points

Present legislation verifies seafood safety according to bacterial indicators of sanitary quality measured through a 5-tube 3-dilution most probable number (MPN) test (Lees, 2000). Besides the wide acceptance, it is recognized that this test presents interpretive,

technical and microbial problems leading to the underestimation of both bacteria indicators and contamination-level and is therefore of limited reliability. The MNP is a statistical estimate of the mean number of bacteria in the sample, thus the result is a semi-quantitative enumeration of bacteria indicators. The precision of the bacteria estimation is low and is dependent on the number of tubes used in the laboratory analysis (Rompré, Servais, Baudart, de-Roubin, & Laurent, 2002). For this reason, this indirect enumeration procedure is intrinsically less accurate than the direct methodologies, unless the population densities are low. MPN method is time-consuming due to the duration of the incubation; it is also tedious and laborious (Hackney, Ray, & Speck, 1979; Rompré et al., 2002). The accuracy of this method is further significantly reduced by the interference of antagonistic bacteria, a certain degree of heterogeneity of the coliform group, the inhibitory nature of the media and weak detection level of slow-growing, stressed or viable or active but nonculturable microorganisms (Rompré et al., 2002). Nonlethal injury may be caused either by temperature, pH, water activity, irradiation, sanitizers, starvation or by a combination of these factors (Hackney et al., 1979). Specially developed media with the appropriated composition may help to recover these stressed or injured cells. Some advantages of this method are: its simplicity, low cost and no need of sophisticated laboratory and equipment. Improvements to the MPN test have been developed over the years. Biochemical tests, based on metabolic reactions, can be used for culturable bacteria identification and enumeration. However, they are not totally specific, and supplementary confirmation tests are necessary. Microbial enzyme profiles can be used to detect indicator bacteria as a complement or alternative to the classical method (Rompré et al., 2002). Nevertheless, innovative methods of bacterial detection and quantification are needed. Molecular methods have appealing characteristics such as sensitivity, specificity, the short time needed to produce results and the fact that they do not require complex culture or additional confirmation procedures, thus allowing for the detection of both culturable and nonculturable bacteria (Hernroth et al., 2002; Pina et al., 1998; Rompré et al., 2002). Additionally, they allow for the detection of more than one microorganism or molecular marker with a single assay (multiplex-PCR) (Scott et al., 2002). Polymerase chain reaction (PCR) or reverse transcriptase-PCR (to detect RNA viral genomes, such as those from viruses) is the most frequently applied nucleic-acid-based method (Le Guyader et al., 2007; Pina et al., 1998; Rompré et al., 2002; Shumway & Rodrick, 2009). Despite the success of PCR and reverse transcriptase (RT)-PCR in detecting minimal starting quantities of nucleic acid (as little as one cell equivalent), the drawbacks of PCR-based assays included low amplification due to the presence of inhibitor substances, and the absence of information about the physiological activity of the bacteria or viruses being studied, because nucleic acids are extracted from viable, dead, culturable or nonculturable microorganisms (Rompré et al., 2002). Some attention must also be paid to results given by methods based on PCR amplification of viruses because they might overestimate the risk for transmission of viable viruses. In addition, molecular approaches can only be performed with highly skilled staff in specialized laboratories providing high-technology services (Hernroth et al., 2002; Le Guyader et al., 2007; Rompré et al., 2002). Real-time PCR overcome the lack of quantification in molecular methods by measuring PCR product accumulation through a dual-labeled fluorogenic probe (i.e., TaqMan Probe). As this method does not require post-PCR sample handling, it also avoids potential contaminations of the PCR product. Real-time quantitative PCR is extremely accurate, reproducible and less labour-intensive than other quantitative PCR methods that also had been designed, and can be applied to both virus and bacteria (Heid, Stevens, Livak, & Williams, 1996).

6. Emerging perspectives

The emergence of *Vibrio* spp. as a human pathogen is of particular concern for shellfish producers. In addition, bivalves contaminated with these bacteria are difficult to recognize since they are not affected in appearance, palatability or smell. Several elimination methods have been proposed: UV depuration, gamma radiation, heat, cold temperatures, tabasco sauce and other horseradish-based sauces. Regardless of their success and limitations, these processes do not represent an alternative for raw seafood (Shehane & Sizemore, 2002).

Bacteriocins (plasmid-derived proteins used as microbial defense systems) have been studied as a method for the removal of *Vibrio* spp. from seafood (Riley & Wertz, 2002; Shehane & Sizemore, 2002). Three bacteriocin-producing strains (IW1, BC1 e BC2), belonging to the group IV bacteriocins of lactic acid bacteria, have been found to exhibit a varied inhibitory spectrum and stability. Bacteriocin IW1 neutralized few strains of *V. vulnificus*, BC1 eliminated several strains of *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* and, finally, BC2 neutralized *Vibrio* spp. *Plesiomonas shigelloides* and *E. coli*. Taking into account both the broadest inhibitory spectrum for *Vibrio* spp. and bacteriocin stability, BC2 was proposed as a new method of control of *Vibrio* spp. (Shehane & Sizemore, 2002).

Bacteriocins have been also investigated as an alternative solution to contamination by *L. monocytogenes*. A large number of IIa class bacteriocins were proposed as highly active against these bacteria (Riley & Wertz, 2002).

Bacteriocins have numerous applications as controlling agents in food but the US FDA only recognizes some bacteriocins as safe for the production of fermented foods such as Nisin, a bacteriocin produced by lactic acid bacteria (Riley & Wertz, 2002; Shehane & Sizemore, 2002). Despite their relatively narrow spectrum of activity against specific bacterial pathogens, bacteriocin's use for the preservation of food creates the dilemma of selecting resistant strains or cross-resistant strains (Riley & Wertz, 2002).

Naturally occurring bacteriophages have been used as biocontrol agents in aquatic environments for fish diseases and other infections (Nakai & Park, 2002). It has been suggested that phage treatment could be useful in controlling *Vibrio splendidus* infection (Sugumar, Nakai, Hirata, Matsubara, & Muroga, 1998) in cultured larvae of the Pacific oyster (*C. gigas*) (Park & Nakai, 2003). Berthe (2005) suggested bacteriophages for the treatment of bacterial infections in molluscan aquaculture production (Berthe, 2005). Although these reports focus on bivalve's pathogens, a similar application could be given to human pathogens. However, reports on microbial control with phages are not available for any bivalve specie or bacterial infection.

Due to the drawbacks associated with obtaining edible shellfish, additional post-harvest processing methods are also being investigated as an alternative for ensuring shellfish safety for human consumption. Since 1992, high pressure processing (HPP) has been proposed as a physical method for food preservation and has already found several commercial food applications, including oyster processing (Murchie et al., 2005). HPP technology makes the inactivation of numerous microorganisms possible by exposing molluscan shellfish to relatively high hydrostatic pressure, for a short period of time at ambient temperatures, while retaining the raw taste, appearance, texture and nutritional properties of the raw shellfish. The same process can be used for shucking oysters without any mechanical force (Kingsley, Holliman, Calci, Chen, & Flick, 2007). These characteristics favour both the shellfish processing industry and consumers. Even though HPP treatment offers advantages over conventional processing techniques in enhancing food safety, the protection is dependent on the composition of food and on the target

microbiota. Microorganisms can differ widely in their intrinsic sensitivities to HPP (Murchie et al., 2005). There is experimental evidence that *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* are reduced by HPP (Calci, Meade, Tezloff, & Kingsley, 2005; Murchie et al., 2005). However, other bacteria reveal a wide range of resistance to HPP depending on the strain (Gram-negative bacteria are, generally, more susceptible than Gram-positive species), growth phase, growth temperature and the composition of surrounding matrices (Murchie et al., 2005). Reports on the use of HHP treatment on raw shellfish showed a reduction of infectious HAV (Calci et al., 2005). However, similarly to bacteria, viruses also differ widely in their vulnerability to HPP (Murchie et al., 2005). Algal toxins will probably be less affected by HPP, but further studies are needed.

The efficiency of HPP-inactivation of microorganisms in shellfish needs further investigation that must include different internal locations of bacteria and viruses in the bivalve, the seasonal and geographical variations in shellfish physiology and composition and lastly the isotonic strength of the harvest waters. Also, additional investigation is needed to determine the mechanisms of inactivation, the reason for the different resistance of viruses and the potential hazard of sub-lethally injured microbiota that may recover and multiply during subsequent storage and may lead to an over-estimation of microbial inactivation. The effects of HPP on both microorganisms and seafood are highly dependent on processing parameters that also need further investigation (Murchie et al., 2005).

In contrast to the previously mentioned, porphyrins present a distinct way of improving shellfish quality since it is focused on the reduction of water contamination rather than in the bivalve. Porphyrins are compounds of natural origin which, when irradiated, generate some hyper-reactive and highly cytotoxic oxygen species (mainly, singlet oxygen) attacking different cellular components. Recently, porphyrins were synthesized to attack several types of microbial cells. The irradiation of the porphyrin causes mortality of a variety of pathogenic agents including Gram-positive and Gram-negative bacteria and parasites in either the cystic or the vegetative stage. These compounds were pointed as a novel photochemical technique for the treatment of microbiologically polluted aquaculture waters (Magaraggia, Faccenda, Gandolfi, & Jori, 2006).

7. Conclusions

The nutritional and economical value of shellfish is acknowledged worldwide. Similarly, filter-feeding bivalves are well known as efficient transmitters of seafood-born disease. Over a long period of time, the high-risk nature of this product and the underestimation factors, have been well documented in many investigative reports and international agencies.

Preventive measures to enhance the quality of living bivalve shellfish when commercialized have included the monitoring and improvement of the water quality found at the harvesting areas. Nevertheless, bacterial indicators used for shellfish health evaluation were announced, in different reports, as inadequate predictors of the presence of autochthonous bacteria and human enteric viruses. Considering the results of these findings, in order to ensure public health, more accurate indexes of water quality and bivalve microbiological safety are required since they are still not available. Also the predictive value of putative indicators needs further evaluation, as specific disadvantages and contradictory results in their use have been pointed out by past studies. Indeed, the overwhelming findings of these studies suggest that the potential indicators may complement the use of *E. coli* for a better guarantee of sanitary safety. However, the development of a local diagnostic scheme for direct detection and identification of the existing pathogens for monitoring bivalve health is probably a future tendency. Future investigations should address the relationships between indicator microorganisms survival with regard to that of the pathogens they are designed to predict. Further work is required to establish a scientific agreement among those considered potential indicators, or others to be discovered, and also to understand the implications of their introduction into legislation. Different threshold levels necessary to cause illness between pathogenic bacteria and viruses must also be considered.

Conventional methodology, applied to predict the level of contamination by quantifying bacterial indicators, needs to be improved in specificity and reduced in time. Detection by new molecular methods may be more sensitive and specific, which will allow for a faster response to health safety problems. The adjustment of the threshold levels of contamination for bacteria and viruses in relation to the risk of occurrence of disease must also be considered. Methods of detecting several pathogens should be implemented so that the assessment of microbial contamination can be more closely associated with the results produced by epidemiological studies.

Depuration and relaying helps to improve shellfish quality but if prevention of human or animal-induced pre-harvest contamination can be achieved, natural causes will always be present. A better knowledge of the parameters affecting the kinetics of the processes of depuration is still needed. More sensitive, reliable, and universally accepted depuration procedures must be developed, so that standardized methodologies can enable the comparison between the experimental results. Technological advances should also be employed.

Reoccurrence of seafood-borne diseases lead to the investigation of alternative methods to eliminate microbial contamination. Bacteriocins, bacteriophages, HPP and porphyrins may be future approaches to control shellfish microbiological contamination. The increased use of antibiotics for the treatment of disease has lead to the emergence of multi-resistant bacteria, which can be released to the environment re-entering the food chain, and consequently, represent a higher risk to consumers. Particular attention should be given to multi-resistant pathogenic bacteria in order to ensure that present or new indicators will be correlated with pathogen occurrence and that methodologies assure the elimination of these bacteria.

Consumer protection involves both the knowledge of the risk associated to the ingestion of raw shellfish and the preventive actions that take into account shellfish specificity, shellfish contamination and adequate regulations. The combination of new depuration approaches and a more accurate quality assessment will help to relieve public concern regarding foodborne diseases associated with shellfish products.

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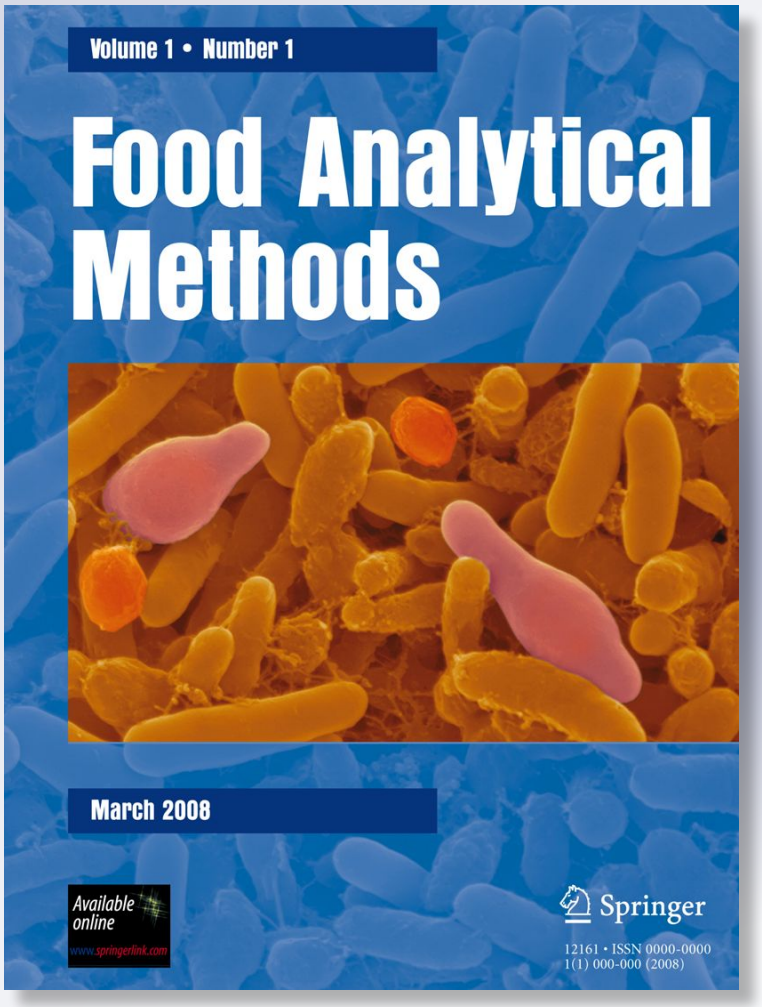

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Comparison of Methodologies for the Extraction of Bacterial DNA from Mussels—Relevance for Food Safety

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Abstract The control of the microbiological quality of bivalve molluscs assumes particular importance because they are among the most produced seafood products and mostly consumed as a whole, raw, or lightly cooked. The composition of the bacterial community associated with bivalves depends mostly on the microbiology of the surrounding environment at growing sites. Once the relationship between microbiology of bivalves and environment is established, a better classification and monitoring of the shellfish beds and evaluation of depuration strategies can be achieved. In this work, we tested if the methods of DNA extraction commonly used for the culture-independent microbiological analysis of sediment and water could be used directly, or with modifications, for the analysis of bacteria in mussels. The commercial kits Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), UltraClean™ Soil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA) and the method described by Griffiths and collaborators for DNA/RNA co-extraction were compared. The efficiency of extraction was assessed by DNA fluorescence and the denaturing gradient gel electrophoresis gel patterns of 16S ribosomal RNA gene fragments were used to compare the reproducibility and representativeness of the extraction methods. Results showed that the DNA/RNA co-extraction method with modifications was the most suitable. However, the results must be interpreted in the light of the purpose of the study and the relevance of maximizing extraction yield or diversity estimate, without compromising reproducibility. To our knowledge, this was the first attempt to transpose the procedure currently used for DNA extraction from sediments or waters, to the analysis of whole mussels.

Keywords Bacterial DNA extraction · Mussels · DGGE · Food safety

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Introduction

Molluscan shellfish are the second largest aquaculture product worldwide, and the blue mussel (*Mytilus edulis*) is among the most widely produced species (FAO 2010).

Bacterial contamination is one of the main constrains to this economic activity (Huss et al. 2004). By filter-feeding nutrients from the surrounding environment, bivalves bioaccumulate autochthonous or anthropogenic bacteria, being extremely susceptible to contamination (WHO 2010). Considering that the traditional cooking procedure of bivalves (lightly cooked) may not completely ensure the inactivation of microbes or toxins, bivalves are critical items in terms of food safety (Lees 2000; Murchie et al. 2005; Romalde et al. 2002). Therefore, the control of the microbiological quality of these products and the classification of growing areas according to sanitary quality (Anonymous 2010) assumes particular importance (Pinto et al. 2006). The presence of

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indicator bacteria (*Escherichia coli*) in concentrations above microbiological standards in bivalve flesh and intravalvular liquid (FIL) may predict the presence of pathogenic bacteria (Anonymous 2004a, b; Anonymous 2005; Anonymous 2007; Anonymous 2008), which implies that the product does not meet the requirements for human consumption and triggers the interdiction of growing areas with consequent economic losses to this sector of activity (Silva and Batista 2008). Also, the decision on the need of depuration procedures, prior to commercialization, is based on the levels of contamination at the growing beds (Oliveira et al. 2011).

Conventional methodology applied to assess microbiological quality of bivalves involves mainly cultivation procedures, using enrichment broths followed by the isolation of colonies on selective media, and confirmation with differential media and biochemical tests (Gugliandolo et al. 2010). Although useful, these techniques are laborious, time-consuming, and susceptible of producing false-positives (Oliveira et al. 2011; Hernández-Zárate and Olmos-Soto 2006; Wagner et al. 1993; Ward et al. 1990; Hugenholtz et al. 1998). In recent years, 16S ribosomal RNA (rRNA) gene sequences have been extensively used for detection, identification, and quantification of bacteria in different samples, namely environmental matrices and food products, by polymerase chain reaction (PCR)-based methods (Hernández-Zárate and Olmos-Soto 2006; Thompson et al. 2005). These approaches have often the advantage of being more rapid, sensitive, and specific, being independent of bacteria metabolic activity, culturability, or pathogenicity (Rompré et al. 2002). These advantages are particularly interesting when risk assessment is conducted in the perspective of human health. Considering that many bacteriological or viral agents that can contaminate mussels produce the first signs of illness in a short time (24–48 h), a rapid response is required so that the cause can be established and geographically localized, and the occurrence of outbreaks can be efficiently prevented (Oliveira et al. 2011; Gugliandolo et al. 2010).

Taking into account that filter-feeders accumulate bacteria from the surrounding environment, the parallel monitoring of the bacterial communities in the sediments, water, and in whole molluscs would provide a more informative approach. Several methods for the extraction of bacterial DNA from sediments and water for metagenomic analyses are currently available in scientific literature. However, to our knowledge, the applicability of these procedures to the extraction of microbial DNA from whole molluscs in order to obtain information susceptible of comparative analysis of the associated bacterial communities has not been tested.

We intended to assess which culture-independent approach of DNA extraction could be used to analyze bacterial communities associated to whole mussels allowing future comparison of their microbial communities to those of sediment and waters. Robust DNA extraction procedures commonly used for the analysis of complex microbial communities in sediment/water samples were adapted and compared for the extraction of microbial DNA from whole mussels, taken as model-bivalves for this study. The results were analyzed in the perspective of achieving good extraction yields, high reproducibility, and the widest possible representativeness of bacterial diversity.

Materials and Methods

Sampling Site and Collection of Mussels

The study area was Ria de Aveiro, a multi-estuarine ecosystem located on the Northwest coast of Portugal (Fig. 1). Pollution of Ria de Aveiro is mainly derived from diffuse drainage of domestic sewage and to harbor and industrial activities, aquaculture farms, and run-off from agriculture fields (Henriques et al. 2004). For this study, *M. edulis* (blue mussels) were collected from Ílhavo Channel (40°35′17″N, 8°41′9″W). This location was chosen because it represents the largest persistent and highly exploited area of mussel cultivation within the estuary (Fig. 1), and it is chronically affected by naturally occurring and anthropogenic microbial contamination being classified as a C zone ([4,600; 46,000] *E. coli*/100 g FIL) (Silva and Batista 2008; Campos and Cachola 2006). Mussel specimens were hand-collected with the aid of a small rake, transferred to sterile bags, and then refrigerated at 4 °C during transport to the laboratory. Analyses were conducted within 3 h after collection.

Mussel Preparation

The shells of intact mussel specimens were scrubbed, washed in tap water, and then thoroughly rinsed with Mili-Q sterilized water. Mussels shells were aseptically opened, and a total of 25 g of FIL were removed and collected on a sterile beaker to which a volume of 250 ml of sterile 8.5% saline solution (Sigma-Aldrich, Co., St. Louis, MO) (1:10) was added. The mixture was homogenized with a Stirlmixer 12 (PBI international, Milano, Italy) at 11,000 rpm for 20 s (Croci et al. 2001). This homogenate was used for all the extraction treatments. Replicates of 30 ml were collected after agitation of the homogenate. Triplicates for each method were randomly chosen from the batch of 30-ml replicates. Homogenates were centrifuged in a Beckman Avanti^{TM} Centrifuge J25I with rotor JA-25.50 (Beckman Coulter, Fullerton, CA) at 1,000 rpm for 5 min for removal of fragments of mussel tissues. The supernatants (25 ml) were centrifuged at 10,000 rpm for 20 min in a Beckman

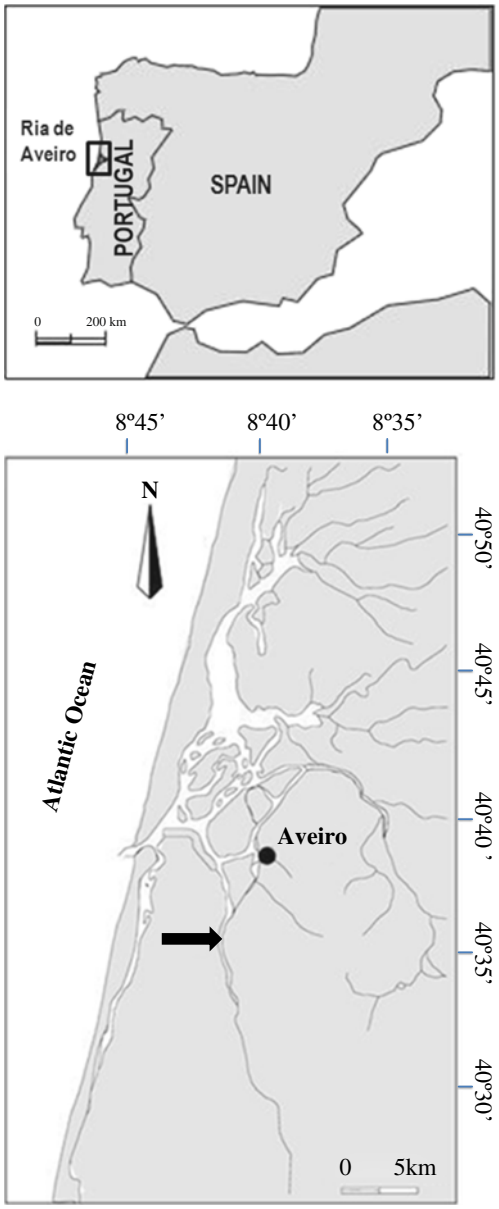

Fig. 1 Ria de Aveiro lagoon (Portugal) with sampling site indicated by an *arrow* (adapted from (Henriques et al. 2004))

Avanti™ Centrifuge J25I with rotor JA-25.50 (Beckman Coulter, Fullerton, CA) for bacterial biomass precipitation, and the liquid phase was discarded. The pellets were resuspended in 1.5 ml of 96% ethanol (Sigma-Aldrich, Co., St. Louis, MO) for preservation until later DNA extraction, PCR amplification, and denaturing gradient gel electrophoresis (DGGE) analyses.

DNA Extraction

For DNA extraction, the material preserved in ethanol was collected by centrifugation at 13,000 rpm for 15 min using a Sigma 1–14 Centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany). DNA was then extracted by three distinct methods: (a) with the commercial Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), usually used for water and tissue matrices, used according to Henriques and collaborators (Henriques et al. 2004); (b) by the DNA/RNA co-extraction method proposed by Griffiths and collaborators (Griffiths et al. 2000), commonly used for sediment matrices; (c) with the commercial UltraClean™ Soil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA). These extraction methods were selected envisaging the possibility of their parallel application to molluscs, water, and sediments from the growing areas allowing future comparison of the corresponding bacterial communities.

DNA Extraction with Genomic DNA Purification Kit

The pellet (0.1 g) was resuspended in 400 µl Tris–EDTA (TE) buffer containing 10 mg ml^{-1} of lysozyme (Sigma-Aldrich, Co., St. Louis, MO) followed by incubation for 1 h at 37 °C in order to achieve chemical disruption of bacterial cells (Henriques et al. 2004). DNA was extracted and purified using the commercial Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), according to the instructions of the manufacturer. DNA was dissolved in 50 µl TE buffer (pH 7.4) (Sigma-Aldrich, Co., St. Louis, MO) and stored at −20 °C until further use. DNA obtained by this method will be hereafter referred to as F-DNA.

DNA/RNA Co-extraction Method

Nucleic acids (DNA and RNA) were extracted from 0.1 g of pellet according to Griffiths and collaborators (2000). DNA obtained by this method will be hereafter referred to as G-DNA.

DNA Extraction with UltraClean™ Soil DNA Isolation Kit

DNA was extracted from 0.1 g of pellet with the UltraClean™ Soil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA) following the instruction of the manufacturer and will be hereafter referred to as M-DNA.

Modifications on the Preparation of Mussel Material Prior to the DNA Extraction Methods

The F-DNA, G-DNA, and M-DNA methods were also performed after introducing some initial modifications which were intended to improve cell lysis.

The pellet (0.1 g) was resuspended in 400 µl TE buffer and submitted to physical treatment by imposing six freeze–thaw cycles (40 s at −196 °C with liquid nitrogen and 2 min at 55 °C with dry heat) and other six cycles of freeze–thawing, including 3 min of ultra-sounds for each cycle.

For G and M methods, the pellet (0.1 g) was collected by centrifugation at 13,000 rpm for 15 min using a Sigma 1–14 Centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany) for further extraction. Extraction of nucleic acids was then performed as previously mentioned. DNA obtained using these modifications prior to the respective extraction method will be hereafter referred to as mF-DNA, mG-DNA, and mM-DNA.

DNA Purification

Nucleic acids obtained by the different procedures were purified with the GENECLEAN® SPIN Kit (Qbiogene, Inc., Carlsbad, CA) according to the instructions of the manufacturer in order to remove impurities and reagents introduced during extraction, prior to downstream uses of the extracted DNA.

DNA Quantity and Purity

DNA quantity and purity were assessed with the NanoDrop® ND-1000 v 3.3 spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE, USA). Alternatively, the DNA quantity was determined by using the Qubit® Fluorometer (Invitrogen™, Life Technologies™, UK) with the Qubit® dsDNA BR Assay Kit (Invitrogen™, Life Technologies™, UK) according to the instructions of the manufacturer.

Amplification of 16S Ribosomal RNA Gene

The DNA extracted was used to amplify the bacterial 16S rRNA gene fragments by a nested approach. In the first PCR were used 0.25 μM of each of the universal primers 8/27f and 1492/1512r (Integrated DNA Technologies, BVBA, Munich, Germany) (Weisburg et al. 1991). The PCR reaction mixture contained 1 μl of template DNA, 3.75 mM $MgCl_2$ (MBI Fermentas, Vilnius, Lithuania), 0.2 mM deoxynucleoside triphosphates (MBI Fermentas, Vilnius, Lithuania), 1× PCR buffer (MBI Fermentas, Vilnius, Lithuania), and 1 UTaq polymerase (MBI Fermentas, Vilnius, Lithuania). Reaction was performed in a MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Denaturation of the template for 5 min at 94 °C was followed by 35 cycles of 1 min at 94 °C, 1 min at 56 °C, and 2 min at 72 °C. The final extension lasted 10 min at 72 °C.

For DGGE analysis, a 410-bp rRNA gene fragment was amplified with primers F968-GC-clamp and R1401 (Integrated DNA Technologies, BVBA, Munich, Germany) (Nübel et al. 1996) using as template 1 μl of the product obtained from the first PCR. Amplification was conducted in a MyCycler™ thermal cycler (Hercules, CA, USA). Additionally, the reaction mixture contained 4% acetamide (Sigma-Aldrich, Co., St. Louis, MO), 0.2 mM deoxynucleoside triphosphates P (MBI Fermentas, Vilnius, Lithuania), 1× PCR buffer (MBI Fermentas, Vilnius, Lithuania), 0.1 μM of each primer, 3.75 mM $MgCl_2$ (MBI Fermentas, Vilnius, Lithuania), and 1 U Taq polymerase (MBI Fermentas, Vilnius, Lithuania). After 4 min of initial denaturation at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 53 °C and 1.30 min at 72 °C were performed. Finally, an extension step at 72 °C for 10 min was carried. Negative and positive controls were included in all reactions.

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

DGGE was performed with the DGGE-2401 system (C.B.S. Scientific Co., Del Mar, CA, USA). PCR amplification products were loaded onto 6.5% acrylamide gels using a denaturing gradient ranging from 22% to 58%, where 100% denaturant corresponded to 7 M urea (VWR International, LLC, Radnor, PA) and 40% (*v/v*) formamide (Sigma-Aldrich, Co., St. Louis, MO). A marker composed by 11 bands was included in the extremities of each gel (Heuer et al. 2002). The electrophoresis was performed at 58 °C for 6 h at 220 V in 1× Tris–acetate–EDTA buffer (5 Prime, Hamburg, Deutschland). After electrophoresis, the gels were stained and fixed for 20 min with 0.3 g silver nitrate (Sigma-Aldrich, Co., St. Louis, MO) in 0.1% (*v/v*) ethanol 96% (Aga S. A., Loures, Lisboa) and 0.005% acetic acid (Merck KGaA, Darmstadt, Germany), rinsed, and then submerged in a developing solution of 0.003% (*v/v*) formaldehyde 37% (Sigma-Aldrich, Co., St. Louis, MO) and 0.33% sodium hydroxide (9%) (Merck KGaA, Darmstadt, Germany). Finally, 0.75% Na_2CO_3 was added to stop the development, and the gel was scanned using a Molecular Image FX apparatus (Bio-Rad, Hercules, CA, USA).

Analysis of DGGE Patterns and Statistical Analysis

The analysis of the DGGE gel was done using Gelcompar 4.0 program (Applied Math, Ghent, Belgium) software package (Smalla et al. 2001). The band profiles from each treatment were exported to a spreadsheet as previously described (Gomes et al. 2010). The band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane, construing a binary matrix. Analysis of DGGE profiles was performed using the PRIMER v5 software (Primer-E Ltd., Plymouth, UK) (Clarke and Gorley 2001, 2006). In the DGGE profiles, the number and precise position of the bands were used as an estimation of the number of different ribotypes present in the community (Miller et al. 1999; Muyzer et al. 1993). DGGE patterns were examined using the Shannon–Weaver index of diversity, H' (Shannon and Weaver 1963), calculated as follows: $H' = -\sum(n_i/N)\ ln(n_i/N)$, where n_i is the relative surface

intensity of each DGGE band and N is the sum of all the surfaces for all bands in a given sample. Statistical significance of variance of the means of the Shannon–Weaver index was evaluated with a one-way analysis of variance. The binary matrix was then transformed into a similarity matrix using the Bray–Curtis similarity index considering band position and intensity. Multivariate analyses of DGGE profiles using this similarity matrix included: (1) cluster analysis, obtained by grouping each DGGE profile according to their similarity (Ramette 2007); (2) analysis of similarities (ANOSIM) tested using 999 permutations, which aimed to determine differences between the treatments compared with variation within treatments, testing the null hypothesis that there are no differences ($R=0$) (*R test* value generally ranges from 0 to 1, where higher values correspond to higher differences—$R \leq 0.25$, no significant differences; $0.25 < R < 0.50$, low significance, moderate separation; and $0.50 < R \leq 1.00$, high significance, high similarity (Clarke and Gorley 2001; Ramette 2007; Chapman and Underwood 1999); (3) multidimensional scaling (MDS) diagram, a two-dimensional map with artificial x- and y-axis was constructed so that each DGGE profile is placed as one point, and similar profiles are plotted together (stress values range from 0 to 1, revealing the reliability of the results for lower values) (Ramette 2007).

Results and Discussion

DNA Extraction Yield

Underlying the objectives of this work was the assumption that, if the same method of extraction of bacterial DNA could be used in molluscs, sediments, and water of harvesting/production sites, a very straightforward assessment of the microbial loads of these areas could be rapidly achieved, also providing the basis for the decision on eventual classification of harvesting areas and depuration strategies. For that, protocols currently used for extraction of nucleic acids from sediment, water, and animal tissues were tested with and without specific modifications. Whole mussels were used as samples for a direct extraction considering that bivalves are eaten as a whole. Bioaccumulation of some pathogens may occur especially in the digestive tube (Power and Collins 1990), but they may also extend to other tissues and be infectious even in very low numbers, constituting a higher risk to public safety (Lees 2000; Richards 1988). Also, this approach is less laborious because dissection is not required. To our knowledge, this is the first attempt to extract bacterial DNA from an entire bivalve.

A purification step was required prior to downstream use of bacterial DNA, in order to minimize the influence of compounds that interfered in PCR (data not shown). This step was applied to all methods ensuring that all subsamples were in the same conditions for further analysis. DNA yields corresponding to the different extracted protocols tested in this work are summarized in Table 1. The yield of DNA extraction by mF method was the highest by both UVQ and fluorescence-based quantification (FQ). However, some DNA from the blue mussel itself may have contaminated the bacterial DNA. This effect may also be present in the modified methods because of the additional mechanical disruption of bivalve tissues (Joanne et al. 1995). The yield of DNA extraction inferred from the fluorescence-based quantification decreased according to the following order: mF, F, mG, G, mM, and M methods. Higher values were obtained by extraction methods including the modification corresponding to the preliminary freeze–thaw sequence.

DNA Purity

The UV absorbance (A260/A280 ratio) was used to assess DNA quality (Fig. 2). The deviation from the theoretical value of approximately 1.8 for DNA extracts indicates the presence of protein, phenols, or contaminants absorbing strongly in the 280 nm region. The extracts obtained by the tested protocols presented a range of 280/260 ratios of 0.93 to 2.09 (Fig. 2), indicating some contamination with RNA in the mF treatment, and with protein or phenol in the treatments M and mM. It has been reported that beadbeating, polyethelene glycol (PEG) precipitation, and hexadecyltrimethylammonium bromide (CTAB) treatment steps generally improve DNA purity (Cullen and Hirsch 1998; Lai et al. 2006). These steps are included in the DNA/RNA co-extraction method (G) which, according to the 280/260 ratio, produces the highest level of purity among the tested procedures.

PCR is a highly sensitive, specific, and rapid method for detecting bacterial gene sequences in pure cultures and

Table 1 Comparison of the extraction yield of the different protocols tested for the extraction of bacterial DNA from whole-bivalve (*M. edulis)* material

Extraction protocol	DNA yield (μg/g) (*M. edulis* sample)	
	UVQ[a]	FQ[b]
Fermentas kit (F)	21.48±1.22	5.69±0.37
Modified Fermentas kit (mF)	80.42±0.58	27.13±0.62
MOBIO (M) kit	0.73±0.59	0.23±0.10
Modified MOBIO (mM) kit	4.58±1.43	1.82±1.41
DNA/RNA co-extraction (G)	8.75±0.32	4.97±0.17
Modified DNA/RNA co-extraction (mG)	9.55±0.60	0.47±0.30

[a] DNA yield based on NanoDrop® quantification

[b] DNA yield based on Qubit® dsDNA BR Assay Kit quantification

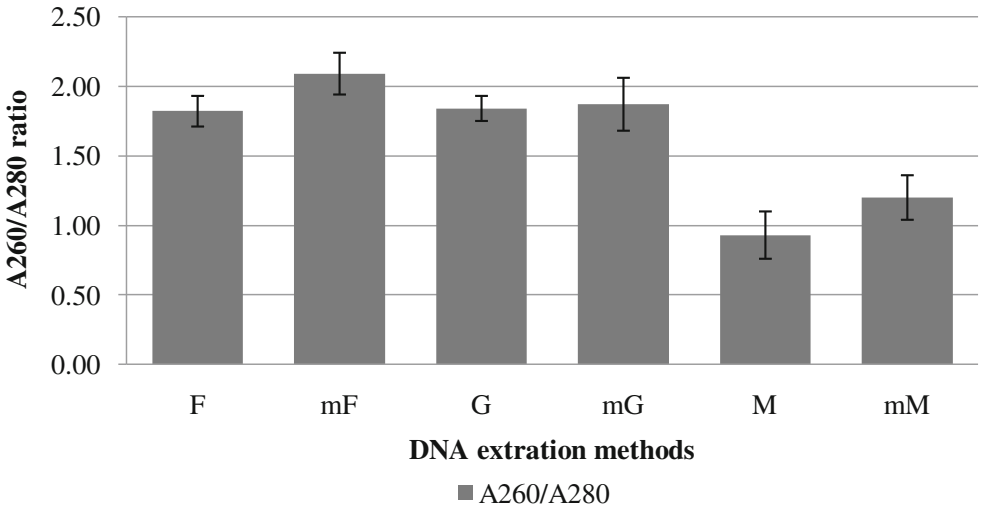

Fig. 2 Ratio A260/280 as an estimate of the purity of DNA obtained by three different extraction methods, with and without the initial freeze–thaw and ultra-sound modification. The values are expressed as the average of three measurements. Error *bars* represent the standard deviation

natural waters. However, when applied to food samples, the amplification reaction can be inhibited or its sensitivity reduced severely (Joanne et al. 1995) by the presence of compounds from the food matrix. Therefore, in addition to quantity, the quality of the retrieved DNA is of major importance for the application of downstream methods of molecular microbiological analysis.

PCR reaction using templates of DNA obtained by the different methods were not consistent in the degree of amplification. In fact, amplification failed for F-DNA and for one of the replicates of mF-DNA (mF-c), although both methods achieved a good extraction yield and only a slight contamination of mF-DNA with RNA could be inferred from the A280/A260 ratio. This may indicate the presence of PCR inhibitors (Rossen et al. 1992) in the extracts obtained by the F or mF procedures.

Representativeness of the Bacterial DNA Extracts

DGGE gel patterns were used for the comparison of the three extraction methods and corresponding modifications used to obtain bacterial DNA from whole mussels. The DGGE profiles are represented in Fig. 3. Differences in DGGE band patterns were clearly visible for the different extraction methods. The number of bands per lane ranged from nine to twenty-five in the mF and mG treatments, respectively. Some bacterial ribotypes, represented as dark bands could be identified in all treatments (e.g., bands 1 and 2) and were interpreted as representing highly abundant bacteria. Other bacterial ribotypes (e.g., bands 3, 4, 5, and 6) were only present in the extracts corresponding to some of the treatments, and most samples produced higher numbers of weak bands, near the limit of detection, which were assumed to represent less abundant ribotypes. The bivalve acts as a colonizing area for the microorganisms present in the immediate environment, in which diversity may vary considerably within very small spatial scales (Gillan et al. 1998). Also, low-intensity DGGE bands may represent less abundant 16S rRNA sequences that may be more affected by extraction and PCR biases than the common and abundant sequences.

The diversity of ribotypes in each DGGE profile was estimated by using the Shannon–Wiener index (H′) presented in Table 3. The DNA yield and quality could explain, to some extent, the diversity of the bacterial community represented in the DNA extract. DNA obtained with the F protocol could not be amplified, and mF method produced the lowest diversity of ribotypes (H'=1.965). The estimated values of bacterial community diversity were highest for mG-DNA. Differences of the diversity of ribotypes obtained by each protocol were not significant, F (4, 9)= 2.159, p=0.155.

Cluster analysis defines groups of DGGE profiles with similar banding pattern/community structure and was used to verify the similarity among replicates of the each extraction methods. The dendrogram representing the cluster analysis (Fig. 4) shows a high degree of variability among replicates. Only the three replicates of the mM method grouped adjacently, with no more than 53% similarity among them. The similarity of the three replicates of the M protocol was 39% while, for the G and mG protocols, it was only 11%. The two replicates of the mF protocol that could be amplified showed a similarity of 55%.

If the presence of high numbers of very weak bands in the DGGE patterns is interpreted as an indication of a community structure where the majority of the frequent ribotypes are present with abundances very close to the detection limit, the modest similarity between replicates may be a consequence of the random presence or absence of a ribotype in each particular replicate.

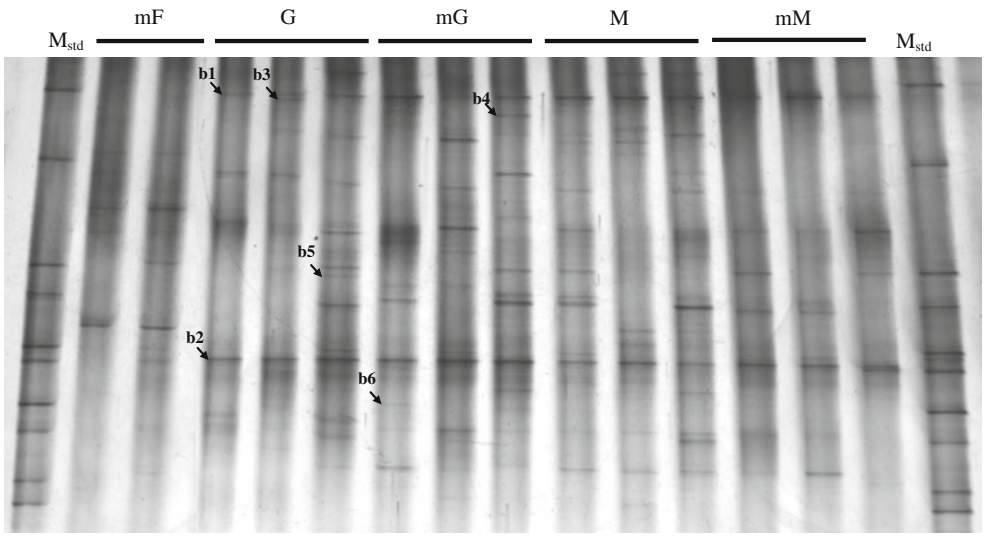

Fig. 3 DGGE profiles of the bacterial community associated to *M. edulis* using different techniques for the extraction of bacterial DNA. DGGE profiles of F-DNA and of replicate mF-c are not presented due to failure in amplification. In the figure, M_{std} marker; *F* DNA extraction method with Genomic DNA Purification Kit, *G* DNA/RNA co-extraction method proposed by Griffiths and collaborators, *M* DNA extraction method with the UltraClean™ Soil DNA Isolation Kit; *m* extraction methods including the modification corresponding to the preliminary freeze–thaw sequence. Replicates of the same extraction method are ordered as follows: *a*, *b*, *c* (*a*, *b* for *mF*)

Considering that dendrograms do not fully represent all pairwise similarities between DGGE patterns, ANOSIM and MDS were used to refine the perspective on the similarity between replicates of each procedure and between procedures. The ANOSIM (Table 2) demonstrated that there are significant differences among profiles obtained from each extraction protocols (global R=0.641). MDS analyses (Fig. 5) corroborate ANOSIM results, indicating that the DGGE profiles corresponding to extracts obtained with the mF method are clearly separated from those obtained with all other treatments (R=1.0). The R value of the pairwise analysis of group mG and G method was negative (R=−0.148) due to the fact that G method contained an outlier replicate (the G-c replicate, as observed in the MDS plot) showing lack of positive correlation between the different replicates (Chapman and Underwood 1999). Indeed, the DGGE profile of the replicate G-c showed a higher diversity (H'=2.980) within the G method (Fig. 3 and Table 3). Furthermore, G replicates showed the lowest similarity (11%) of all the tested protocols (Fig. 4). The DGGE profiles of mG and M protocols did not show significant

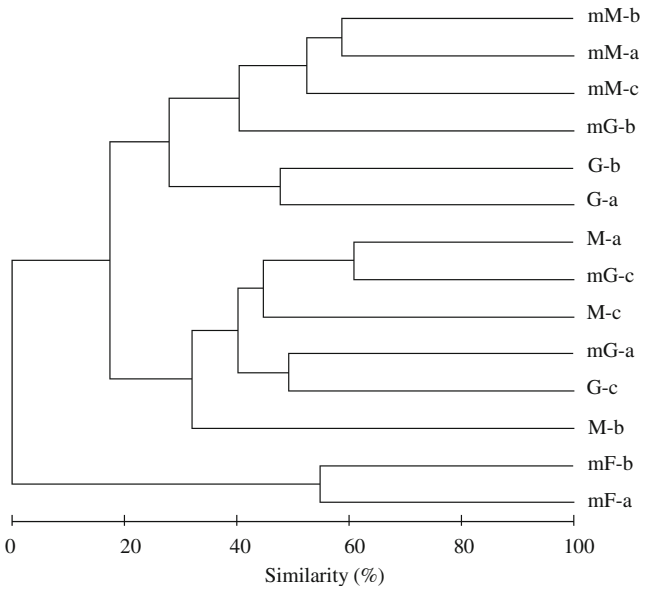

Fig. 4 Dendrogram generated from the patterns of bands obtained by DGGE (Fig. 3). In the figure, *F* DNA extraction method with Genomic DNA Purification Kit, *G* DNA/RNA co-extraction method proposed by Griffiths and collaborators, *M* DNA extraction method with the UltraClean™ Soil DNA Isolation Kit, *m* extraction methods including the modification corresponding to the preliminary freeze–thaw sequence; *a*, *b*, *c* replicates

Table 2 ANOSIM statistics analysis (R) of Bray–Curtis similarity measures generated from the patterns of bands obtained by DGGE (Fig. 3) of DNA extraction method with Genomic DNA Purification Kit (F), DNA/RNA co-extraction method proposed by Griffiths and collaborators (G, and DNA extraction method with the UltraClean™ Soil DNA Isolation Kit (M)

Groups	R Statistic
mF, G	1.0
mF, mG	1.0
mF, M	1.0
mF, mM	1.0
G, mG	−0.148
G, M	0.593
G, mM	0.815
mG, M	0.148
mG, mM	0.556
M, mM	0.519
Global R	0.641

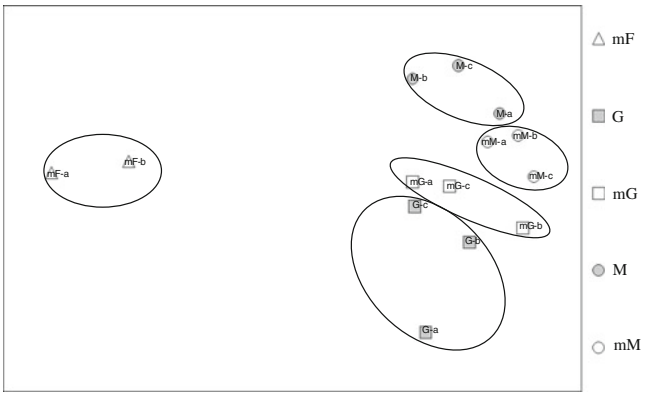

Fig. 5 MDS plot (stress value of 0.13) showing relative similarity between extraction methods, as determined from the patterns of bands obtained by DGGE (Fig. 3). In the figure, *F* DNA extraction method with Genomic DNA Purification Kit, *G* DNA/RNA co-extraction method proposed by Griffiths and collaborators, *M* DNA extraction method with the UltraClean™ Soil DNA Isolation Kit, *m* extraction methods including the modification corresponding to the preliminary freeze–thaw sequence

differences (R=0.148), implying higher similarities within replicates patterns when compared with similarities between methods. In MDS plot, for the G and M protocols, the patterns corresponding to the freeze–thaw modifications are more similar (R=0.556) than the respective non-modified procedures (R=0.593), which clearly produced different banding patterns. This result shows that the freeze–thaw modification enabled the extraction of more common ribotypes in M and G methods. Considering that the freeze–thaw modifications improved bacterial DNA extraction yield from the mussel matrice, the enhanced similarity between mM and mG protocols may result from a better recovery of the more rare ribotypes which are close to the detection limit of the technique and that could easily be missed in some replicates of the standard M or G protocols, making the DGGE patterns less reproducible.

Table 3 Shannon–Weaver index of diversity (H') for each replicate of the different extraction methods

Sample	Diversity (H')	H' Mean	H' Standard deviation
mF-a	1.777	1.965	0.265
mF-b	2.152		
G-a	2.320		
G-b	2.381	2.560	0.365
G-c	2.980		
mG-a	2.370		
mG-b	2.967	2.664	0.299
mG-c	2.654		
M-a	2.488		
M-b	2.207	2.489	0.283
M-c	2.773		
mM-a	2.417		
mM-b	2.467	2.446	0.026
mM-c	2.454		

In the table: *F* DNA extraction method with Genomic DNA Purification Kit, *G* DNA/RNA co-extraction method proposed by Griffiths and collaborators, *M* DNA extraction method with the UltraClean™ Soil DNA Isolation Kit, *m* extraction methods including the modification corresponding to the preliminary freeze–thaw sequence, *a*, *b*, *c* replicates of the same extraction method

Conclusion

The modifications applied to protocols F, G, and M were effective in improving the DNA extraction yield. Some drawbacks, as the presence of non-specific inhibitors in the sample, from the body of the bivalves, must be further analyzed. The effect of sample size (mass of mussel FIL used for the extraction or particular tissues) on the reproducibility of DGGE patterns was not addressed in this work, but it may be relevant in the representativeness of the bacterial DNA for metagenomic analyses. Other sample preparation techniques for PCR-based detection of bacteria in bivalves might be evaluated in the future.

The DGGE profiles were characterized by a high degree of variability between replicates which can be associated to the presence of high number of weak bands (less abundant bacteria), probably very close to the detection limit of the method. The mG method for bacterial DNA extraction was the most suitable for investigation of the bacterial community from the blue mussel *M. edulis*. Despite the rather low similarity between replicates, this method showed a fair DNA extraction yield, a good degree of purity, and provided the more complete representation of the community. Although being slightly more laborious because of the initial preparation of reagents and more time-consuming than the M method because of the multiple steps involved, the mG method may turn out advantageous in view of its simplicity and low cost. It is worth considering that this approach can also be used when RNA-based analysis is intended, simply by skipping the step of DNA purification. Therefore, from the culture-independent approaches of DNA extraction here tested, mG method shows potential for the analysis of bacterial communities associated to whole mussels in the perspective of comparison of the microbial communities from bivalves, sediment, and waters.

The presented research contributes to the development of a convenient approach for the classification of growing areas, assessing the microbiological quality of bivalves and shellfish growing areas by molecular methods and may ultimately provide the practical basis to routine microbiological monitoring ensuring public heath safety.

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Bacteriophage therapy as a bacterial control strategy in aquaculture

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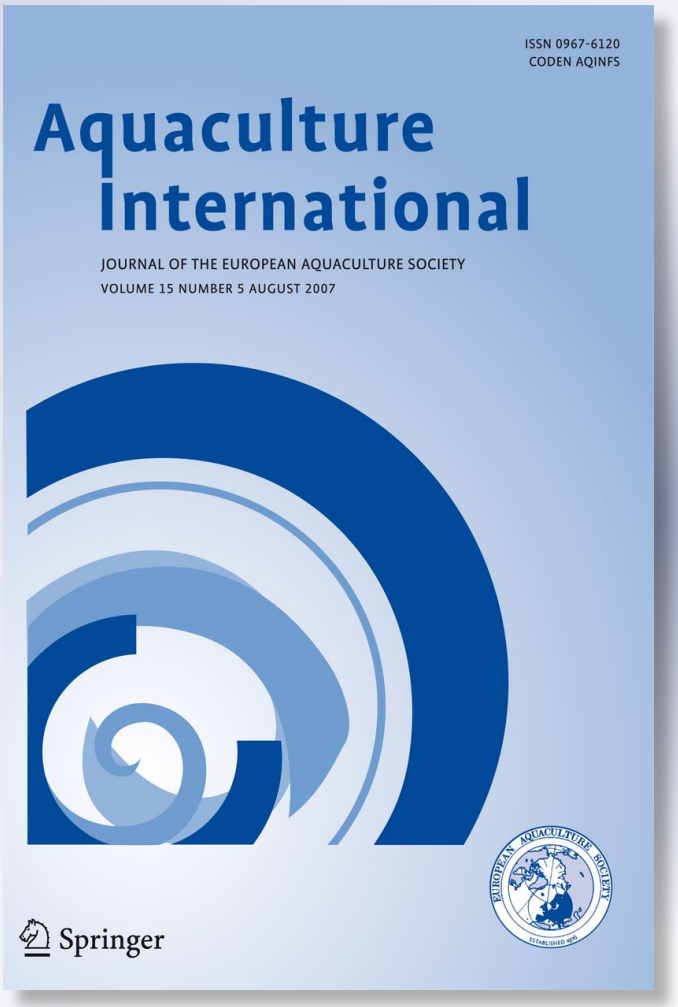

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Bacteriophage therapy as a bacterial control strategy in aquaculture

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Abstract Aquaculture is a sector of economic relevance worldwide. Bacterial infections have been recognized as an important limitation to aquaculture production and trade. Microbial infection in aquaculture derived products has been prevented by antibiotic administration with limited success. Recently, drug-resistant bacteria have become a global problem, urging for the prompt development of alternative control strategies in order to improve food quality and safety. The alternative approach of using lytic phages or their products, as bioagents for the treatment or prophylaxis of bacterial infectious diseases, has gained interest. This review intends to emphasize the need of further research in the field of the application of phage therapy in aquaculture and highlights the use of phages in invertebrates as an antimicrobial strategy pointing critical aspects from the economic, environmental and public health perspectives.

Keywords Bacteriophage · Bacterial infections · Aquaculture · Food safety · Public health

Introduction

Aquaculture is the cultivation of aquatic populations including finfish, shellfish and plants, under controlled conditions such as breeding and confinement, along with supplying

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nutrients and medication, in order to increase production (Pillay and Kutty 2005). Recently, aquaculture production has become one of the fastest-growing animal food-producing industries (FAO 2006, 2009). Between 2004 and 2006, the annual growth rates were 6.1% (in quantity) and 11.0% (in value) (FAO 2009). Despite the international development of this production sector, microbial diseases outbreaks resulted in important economic losses being considered the major problem associated with aquaculture (Hektoen et al. 1995; Berthe 2005). Some of the typical fish farming diseases caused by the main biological agents (bacteria, viruses, parasites and oomycetes) have been briefly reviewed elsewhere (Almeida et al. 2009). Bacterial diseases caused by indigenous and non-indigenous pathogenic bacteria, particularly multiresistant bacteria, are a major issue in aquaculture. Although the administration of antibiotics is approved by authorities and it represents an easy and relative low-cost solution, if compared to the economic loss that bacterial infection causes, some important findings indicate that this strategy has limited success: development of multi-drug resistance in bacteria, microorganism substitution, ecological and public health impacts (Park et al. 2000; Perreten 2005). There is an increasing need for finding alternative ways to control microbial diseases in aquaculture. Recently, bacteriophages gained increased attention as an alternative to antibiotics and other antibacterial chemicals in order to control microbial diseases and prevent the spreading of multiresistant bacteria in aquaculture (Nakai and Park 2002). Bacteriophages were discovered early in the twentieth century, and variable attention has been given to research on bacteriophage applications. Bacteriophages or phages are viruses that specifically interact with their host bacteria through two major infection cycles (Fig. 1). These are the lytic (or virulent) and lysogenic cycles and more sporadically through pseudolysogeny (Ripp and Miller 1997, 1998). Lytic phages replicate inside the host cell and progeny viruses are released causing cell lysis. This capacity to destroy host bacteria sets the stage for the use of lytic phages as therapeutic or prophylactic agents. A method using naturally occurring lytic phages, or their products, as bioagents for the treatment of bacterial infectious diseases is called bacteriophage therapy or phage therapy (Nakai 2010).

This work intends to critically summarize recent literature on the application of phages in aquaculture, indentifying its benefits and potential drawbacks envisaging bacteriophage therapy as an alternative way to control bacterial diseases found in aquaculture organisms (fish, crustaceans and molluscs). We highlight the perspective of using phages in invertebrates, particularly, bivalve molluscs, providing a critical view for further research since little information concerning this antimicrobial strategy in these animals exists.

The need for an alternative to chemotherapy in aquaculture

Like other animals and humans, cultured fish and shellfish are persistently colonized and infected by microorganisms (Nakai and Park 2002). Aquaculture products are highly susceptible to bacterial infections due to non-hygienic and often stressful conditions of cultivation (Muroga 2001; Sapkota et al. 2008). Susceptibility to bacterial pathogens can be partially reduced by some preventive measures: (1) avoiding pathogen transmission among culture batches; (2) improving water quality control; (3) minimizing temperature and salinity alterations; (4) reducing stress by controlling organism densities, handling and nutrition and lastly (5) the introduction of hygiene practices such as the disinfection of tanks, water and eggs (Howgate et al. 1997; Nakai et al. 1999; Jorquera et al. 2002; Defoirdt et al. 2004). Even with preventive measures, chemical anti-infective agents (antiseptics and anesthetics) are still used to reduce susceptibility to bacterial pathogens

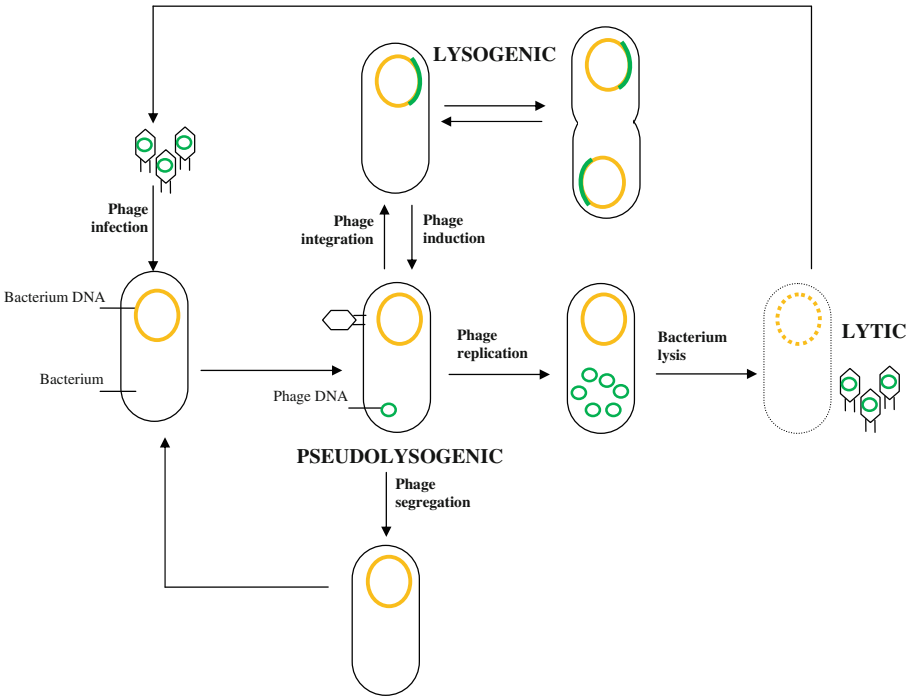

Fig. 1 After phage infection, and if phage segregation does not occur, temperate phages may undergo the lytic or lysogenic cycle. In the lytic cycle, new phages are produced and released during bacterium lysis. In the lysogenic cycle, the phage DNA is included in the host chromosome and replicated along with the bacterial DNA. Occasionally, the prophage may excise from the host genome and the lytic cycle begins. In pseudolysogeny infection, bacteriophage enters a dormant intracellular phase where the phage genome is not integrated into host genome. Bacteriophage can proceed to either lytic or lysogenic infection cycles (Adapted from Griffiths et al. 1999; Sulakvelidze et al. 2001)

(Fauconneau 2002; Defoirdt et al. 2004; Sapkota et al. 2008). Antibiotics are commonly used worldwide for therapeutic purposes (Fauconneau 2002; Sapkota et al. 2008). However, there is a lack of regulatory legislation in the licensing of antibiotics and a need for international harmonization of those available for aquaculture use, which only exist in a limited number (Tan et al. 2000; Fauconneau 2002; Taylor et al. 2002; Morrison and Rainnie 2004; Alanis 2005; Sandeep 2006; Sapkota et al. 2008; Almeida et al. 2009; Daniel 2009). Chemotherapy is not always successful since some antibiotics are administered primarily in food mixtures, and diseased animals show poor feeding habits (Wu and Chao 1982; Morrison and Rainnie 2004). Additionally, environmental and public health problems arise from the use of antibiotics: (1) selection of bacterial resistance to antibiotics due to excessive prophylactic and therapeutic use, especially in sublethal doses (Cabello 2006); (2) the up-contaminations in the food chain related to the transference of resistant organisms between animals and humans, and among animals (Inglis et al. 1991; Inglis et al. 1993a, b; Perreten 2005); (3) microorganism substitution (Park et al. 2000) and (4) the toxicity of most antibiotics and their residues to aquatic organisms and surrounding environment (Cabello 2006). These drawbacks had led to an increased urgency in finding alternative ways to control microbial outbreaks in aquaculture (Nakai and Park 2002; Defoirdt et al. 2004; Perreten 2005; Sandeep 2006; Sapkota et al. 2008). Several

antimicrobial approaches have been proposed for application in aquaculture: disruption of bacterial quorum sensing (Defoirdt et al. 2004; Defoirdt et al. 2007; Bai et al. 2008; Jiang and Su 2009), probiotics (Gibson et al. 1998; Moriarty 1998; Verschuere et al. 2000; Nikoskelainen et al. 2003; Farzanfar 2006), microbial matured water (Munro et al. 1994; Skjermo et al. 1997; Skjermo and Vadstein 1999), antibacterial photodynamic therapy with cationic phthalocyanines and porphyrins (Magaraggia et al. 2006; Almeida et al. 2009), green water systems (Tendencia and de la Peña 2003; Defoirdt et al. 2007), immunostimulants (Vadstein 1997; Bricknell and Dalmo 2005), bacteriocins (Riley and Wertz 2002; Shehane and Sizemore 2002; Schöbitz et al. 2006), polyculture (Tendencia 2007) among others (Defoirdt et al. 2011). The presented work will emphasize the concept of phage therapy to treat bacterial infections in aquaculture systems. Evidence in support of the effectiveness of phage therapy against bacterial infectious diseases associated to diverse areas has been accumulated and is already being applied (Inal 2003; Housby and Mann 2009).

Important considerations for bacteriophage use in aquaculture

The establishment of the causative agent of the disease is the major step in bacteriophage therapy. Then, it is necessary to select the bacteriophages that can effectively infect the target bacteria and to evaluate the potential of those bacteriophages to control bacterial diseases in aquaculture. The procedure involves several sequential steps: (1) isolation of lytic bacteriophages from the fish surroundings, using an enrichment method; (2) culture of the bacteriophages; (3) phenotypic and genotypic characterization of the bacteriophages; (4) bacteriophage typing of the target bacterium and (5) selection of lytic bacteriophages for therapeutic use. Additional work is needed to establish practical procedures for bacteriophages application in aquaculture: (6) assessment of therapeutic efficacy of the phages against experimental infections (in a laboratory) and natural infections (in field trials) and (7) recognition of the existence of virulence genes or other toxic factors in the phage. Finally, phage therapy must be established at a large scale with culture and long-term preservation methods of the phages for commercial development (Nakai 2010).

The use of bacteriophages as bacterial control agents in aquaculture has advantages and drawbacks (Table 1), and some of the critical points are the preparation of phage stocks, the effect in field conditions, the procedure for phage administration, the effect against the natural bacterial community, the potential risk for the transference of genes related to virulence, the development of bacterial resistance to phage infection and aspects related to legal regulation and public opinion.

Phages preparations to be used in aquaculture

High purity of the lytic phages isolates and of the phage stocks is required for phage therapy. The removal of bacterial debris (as endotoxins and lipopolysaccharides) can be easily achieved (Carlton 1999; Inal 2003; Morrison and Rainnie 2004) eliminating further problems (Efrony et al. 2007; Phumkhachorn and Rattanachaikunsopon 2010). Contaminated phage suspensions might be fatal for the organism injected (Morrison and Rainnie 2004). However, this is of less concern in the treatment of most bacterial pathogens of aquatic species if phage suspensions are orally administrated (Morrison and Rainnie 2004).

Characterization of the bacteriophages and bacteriophage typing are necessary before the application of phages to control a particular bacterial pathogen due to the high degree

Table 1 Pros and cons of bacteriophage alternative

Issue	Advantage	Disadvantage	Reference
Abundance	Ubiquous providing a large, naturally available pool of bacteriophages	Strongly lytic phage strains must be selected from the available pool	Morrison and Rainnie (2004)
Multiplication and self limitation	Rapid exponential replication and declining, along with bacterial growth not posing an ecological risk Repeated administration is not necessary	Makes it difficult to extrapolate from in vitro phage growth data to in vivo expectations, to interpret in vivo data and to generalize from one in vivo situation to another	Inal (2003), Weld et al. (2004)
Host specificity	Relatively narrow host range allowing that no other bacteria will be inactivated, namely useful bacteria and the normal intestinal microflora Reduced possibility of secondary infections development and side effects are less likely to occur	The exact host bacterium causing the infection needs to be identified Strain specific rather than species specific, increasing the difficulty when preparing phages for highly diverse bacterial variants	Barrow and Soothill (1997), Carlton (1999), Nakai and Park (2002), Mathur et al. (2003)
Isolation, selection and maintenance	The selection of strictly lytic phages, the sequencing of the hereditary material of phages and toxicity tests can minimize the risk of transference of toxic genes between bacteria	High diversity within populations of both phages and bacteria implying an understanding of their heterogeneity and ecology Needs expertise and an established set up	Yuksel et al. (2001), Wagner and Waldor (2002), Mathur et al. (2003), Brüsso et al. (2004), Flegel et al. (2005), Scott et al. (2007), Stenholm et al. (2008), Nakai (2010)
Bacterial debris	Removal can be readily achieved by current technology	Might cause therapy to fail since it might be fatal for the organism injected	Carlton (1999), Inal (2003), Morrison and Rainnie (2004)
Administration	Through feed impregnated, injection or by immersion allowing treatment of animals at various stages from eggs to broodstock	Poor feeding habits of diseased animals Injections might be unpractical to treat a large number of animals	Barrow and Soothill (1997), Nakai and Park (2002), Inal (2003), Mathur et al. (2003), Nakai (2010)
Dose	Determination of precise initial dose may not be essential since page titers may increase along with bacterial infection	Limited data available on effective phages doses	Inal (2003), Mathur et al. (2003), Nakai (2010)
Fate	Phages decrease after killing the target bacteria being finally excreted and not posing any environmental risk	Dose of administration must account for those phages that are quickly excreted	Barrow and Soothill (1997), Lorch (1999), Phumkhachorn and Rattanachaikunsopon (2010)
Multiple infections	A mixture of phages bringing synergistic effects can be applied	All the infecting bacteria must be exactly recognized	Carlton (1999)

Table 1 continued

Issue	Advantage	Disadvantage	Reference
Bacteria resistance	Overcoming resistance is not difficult attending to the worldwide abundance and to the rapid mutation of phages	Phage-resistant mutants are fairly common and with rapid appearance	Levin and Bull (2004), Merril et al. (2006), Sandeep (2006), Nakai (2010)
	Phage-resistant colonies are not necessarily still pathogenic as selection for resistance could select against virulence	Newly isolated phage requires efficiency tests	
Immunology	A higher dose of phage can compensate for those that are rendered non-viable by interaction with neutralizing antibodies	Phage-neutralizing antibodies might prevent some proportion of the administered dose of phages from being able to adhere to the bacterial target	Carlton (1999), Morrison and Rainnie (2004)
Timing of treatment	The production of phage-neutralizing antibodies is slower than the kinetics of phage action	Chronic treatments may fail due to phage-neutralizing antibodies	Sulakvelidze and Morris (2001, Inal (2003)
Economic costs	Compensated by the therapy efficiency in treating diseases and might be less expensive than that of using antibiotics	Procedures required after phage therapy could become too expensive	Carlton (1999), Matsuzaki et al. (2005), Miedzybrodzki et al. (2007)
		Additional costs due to the need of further research in the field	

of phenotypic and genotypic diversity within populations of both phages and bacteria (Stenholm et al. 2008). The preparation of lytic phages to control highly diverse bacterial strains is critical because phages are usually strain specific. This leads to the need of phage cocktails combining different phages. For instance, *Pseudomonas plecoglossida* is a serologically uniform bacterium and is infected by a single phage type. For other bacteria, such as *Lactococcus garvieae*, a major phage type of broad infectivity exists which can lyse more than 90% of strains isolated from fish. If a given target bacterium is highly variable in its phage types or very changeable in its phage sensitivity, large collections of therapeutic phages with different lytic activities are undoubtedly required in phage therapy practices (Nakai 2010).

Efficiency in aquaculture conditions

Assessing the efficacy of the lytic phages against experimental and natural infections is crucial before phage therapy because bacteriophage with a lytic lifecycle within a well-defined in vitro environment may not remain lytic in vivo (Sandeep 2006). Indeed, the low in vivo activity of phages has been pointed as the reason why little value was attributed to phages in controlling bacterial infections in man and animals (Nakai et al. 1999; Park et al. 2000). The trials for evaluation of the efficiency of the phages in field conditions also serve

the purpose of establishing the dose and route of phage administration. Data available on effective phage doses are limited and mainly related to treatment of fish. However, in contrast to chemicals and other substances, determination of precise initial dose given to individual fish may not be essential in the aquaculture setting, because of the self perpetuating nature of the phages causing phage titers to increase along with bacteria in infected individuals or in pathogen-contaminated water (Nakai 2010).

Administration routes

The administration of phages can be done by impregnated feed or injection (Nakai and Park 2002). Even considering that diseased fish may not feed well, the administration of phages by impregnated feed enables the treatment of a large number of fish specimens. This technique can be advantageous for bacterial infections that occur through an oral route since the intestine is also a main way for the pathogen to enter the organism, and normal intestinal flora might be unaffected, but the target bacteria will be (Nakai 2010). Although an accurate inoculation can also be achieved by injection, this may be laborious when a large number of animals or very small animals require treatment. Nevertheless, this approach is used for a number of vaccines available in the market (Nakai 2010). The addition of therapeutic phages in the water medium is also possible because they are likely to remain effective and stable as if they were in a liquid culture or the medium of their origin (Nakai et al. 1999). This type of administration has the advantage of continuous and intimate physiological contact between the organism infected and the bacteriophage (Summers 2001; Inal 2003). For this reason, the immersion in phage suspensions will be more effective for organisms in which infection is initiated by bacterial colonization of the skin and gills (Nakai and Park 2002). Furthermore, in the comparison of results between the laboratory and the field, the immersion approach allows a higher similarity between environmental and laboratory conditions because phage-bacterium interaction occurs in suspension (Summers 2001). Bathing or immersion techniques have been valuable to treat larvae, juveniles or eggs in hatcheries as shown in the biocontrol of *Vibrio harveyi* in *Penaeus monodon* larvae (Vinod et al. 2006; Karunasagar et al. 2007). The use of multiple routes of phage administration is very advantageous to aquaculture since microbial infections can occur at various stages from eggs to broodstock (Nakai 2010).

Immunitary responses

Phages are recognized by the immune system of animals as external entities and an immunologic response might develop (Pirisi 2000; Sulakvelidze and Morris 2001). Phage-neutralizing antibodies may decrease phage effectiveness in vivo (Pirisi 2000; Sulakvelidze and Morris 2001), and a higher dose of phage might be needed to compensate for those phages that are rendered non-viable by interaction with neutralizing antibodies (Carlton 1999; Morrison and Rainnie 2004). Therefore, in treating chronic or recurrent infections, it appears that one type of phage or mixtures of phages can only be used once for intravenous treatment because of prior exposure (Inal 2003). In order to overcome immune responses, it is important to understand whether phage-neutralizing antibodies are produced and for how long they remain in circulation, which factors of the immune response of the vertebrate host are able to inactivate the phages and lysis and if phage inoculations given too early could be less effective or fail completely (Barrow and Soothill 1997; Yuksel et al. 2001; Payne and Jansen 2003). The production of phage-neutralizing antibodies after phage administration, in aquaculture, is not documented in literature.

For instance, phage-neutralizing antibodies were not detected in yellowtail that repeatedly (successive 7 days) received phage-impregnated feed or in ayu after receiving intramuscular injections of phages (Nakai et al. 1999; Park and Nakai 2003). Immune response does not pose a problem for phage therapy in invertebrates, and this approach has been proven efficient in these organisms (Efrony et al. 2007).

Transference of virulence genes

The presence of virulence genes or other potentially harmful elements by phages must be assessed prior to phage therapy application. Phages may act as vectors for the transference of virulence or resistance genes transforming non-pathogenic bacteria in pathogenic strains (Wagner and Waldor 2002; Scott et al. 2007). It has been estimated that the global rate of phage-mediated genetic modification in bacteria as being up to 20×10^{15} gene transfers events per second (Brüsso et al. 2004). Munro and colleagues demonstrated that the presence of the bacteriophage *V. harveyi* myovirus-like (VHML) could confer virulence to *V. harveyi* strains explaining the large variation in pathogenicity among strains of *V. harveyi*, the causative agent of luminous vibriosis in larval prawns systems (Munro et al. 2003). Two apparently unrelated bacteriophages (one from the family *Myoviridae* and the other from the family *Siphoviridae*) were found to induce increased virulence in *V. harveyi* (Flegel et al. 2005). Bacteriophages may sometimes mediate the toxicity of *V. harveyi* in *Penaeus monodon* by the transfer of a toxin-encoding gene or a regulator gene controlling toxin production (Lila et al. 1999). The possibility of rapid horizontal transfer of virulence factors among a bacterial population upon accidental introduction of a lysogenic phage was pointed as an inherent risk for shrimp farmers (Flegel et al. 2005). Also, public health concerns related to the spread of antibiotic resistance genes arise. For example, *V. harveyi* Siphophage 1 (VHS1) loses the ability to lyse cells but retains its ability to lysogenize after boiling for 10 min, which means that cooking crustaceans may not be sufficient to fully inactivate bacteriophages that might be present in this seafood (Flegel et al. 2005).

Development of bacterial resistance to phage infection

Altered phenotypes resulting from mutations include bacterial resistance to phage infection (Scott et al. 2007). The susceptibility or resistance of bacterial strains to phage lysis is partially due to the variation of receptor molecules or modification of the host restriction system (Shivu et al. 2007). The receptors to which phages are targeted on the bacterial cell surface act as virulence factors so, when bacteria develop resistance to phage, they are usually changed which results in an attenuation of virulence (Barrow and Soothill 1997; Skurnik and Strauch 2006). These phenotype adaptations may be attributed to a change in selective pressure on the bacterium, imposed namely by phages. Phage-resistant colonies are not necessarily as pathogenic as wild types because the selection for resistance may be accompanied by a decrease of virulence (Merril et al. 2006). In chemotherapy, bacteria that develop resistance to antibiotics remain pathogenic, while in phage therapy virulence might be attenuated and phage-resistant bacteria might be less pathogenic. Therefore, the success of phage therapy might also rely on changes in bacteria-phage interaction at the level of the attachment sites (Wagner and Waldor 2002). Research in aquaculture has showed different results with respect to the emergence of phage-resistant bacteria. Although some reports showed no evidence of phage-resistant bacteria as a consequence of phage therapy in diseased fish or apparently healthy fish (Park and Nakai 2003), others demonstrated this effect. The emergence of mutants resistant to phage infections was

demonstrated for *A. salmonicida* although these bacteria could initially be infected by more than one phage (Imbeault et al. 2006). Those mutants that were resistant to one phage were sensitive to a different phage or even more than one phage types. Resistant bacteria had a slower generation time than the original strain and a very low success of replating in tryptic soy agar. All mutants were sensitive to three or more phages, and 25% of the mutants seemed to revert to the original-strain phenotype after a first replating (Imbeault et al. 2006). Phage-resistant bacteria were also detected in the treatment of beta-hemolytic streptococcicosis in Japanese flounder *Paralichthys olivaceus* (Matsuoka et al. 2007). Phage-resistant *Streptococcus iniae* remained pathogenic to fish and was isolated from dead fish in the phage-treated group (Matsuoka et al. 2007). These results bring into question the application of bacteriophages to control furunculosis and streptococcosis.

Multiple drug resistance can emerge as a consequence of phage administration namely by transference of resistance plasmids between bacteria of diverse origins in natural microenvironments (Kruse and Sørum 1994). Newly transformed strains may be more or less susceptible to phages, and there is still the need to find a phage specific for the newly emerged strain. However, phages are abundant and widely distributed in nature and also undergo mutations, some of which can match bacterial genetic changes, restoring affinity (Carlton 1999; Sulakvelidze et al. 2001; Sulakvelidze and Morris 2001; Kutter and Sulakvelidze 2005). The multiple tactics of phages to avoid, circumvent or subvert antiviral mechanisms of bacteria were revised by Labrie and co-authors (Labrie et al. 2010). Bacteria that become resistant to a certain type of phage can still be infected by other phage types that can be isolated from nature (Carlton 1999). The use of formulated mixtures of phages (phage cocktail), phage lysins alone, or the combined use of a phage cocktail and antibiotics are attractive strategies because the efficiency of treatment may increase and the emergence of resistant mutants minimized (Thiel 2004; Petty et al. 2007). However, it has been reported that adjuvant use of an antibiotic could sometimes decrease the efficiency of phage therapy, and combined treatments may enhance the problem of resistance (Mathur et al. 2003; Payne and Jansen 2003).

Some of the measurements to control the emergence of phage-resistant mutants involve: (1) using mutant phage derived from the phage that was initially active against the wild-type bacteria (Carlton 1999; Almeida et al. 2009; Labrie et al. 2010); (2) using a newly isolated phage; (3) using a mixture of different strains of phages that would prevent the emergence of a resistant bacteria during the treatment (Nakai and Park 2002; Thiel 2004; Petty et al. 2007); (4) using different therapeutic strategies in combination or (5) using lysins rather than bacteriophages (Inal 2003; Matsuzaki et al. 2005). The problem of resistant bacteria is critical and needs further research.

Large-scale application of phage therapy: regulatory authorities, public and scientific awareness

The use of phages at the production scale is promising either for obtaining products with reduced bacterial loads or to inactivate microorganisms in the surroundings improving sanitary conditions (Inal 2003). Additionally, the prophylactic use of a phage cocktail could improve the water quality of aquaculture plants by reducing the concentration of the bacteria most common in aquaculture animals (Withey et al. 2005). This is of particular importance considering that bacteria resistant to antimicrobials might be present in microenvironments such as water, pelletized feed (Miranda and Zemelman 2002) and bed sediments (Kerry et al. 1994). However, at present, phage-based therapeutic items have not yet been produced and/or approved for aquaculture use.

Approvals from the United States Department of Agriculture (USDA) and Food and Drug Administration (FDA) (21CFR172.785) were given to the company Intralytix for the use of bacteriophage-based preparations as food additives in ready-to-eat foods. The advantage of this phage additive is that it does not add flavor, aroma or nutritional value to food and is viewed as being a natural and a preservative-free alternative. Approvals were also given by the Environmental Protection Agency (EPA) authorizing the use of a mixture of phages on food-handling surfaces (EPA registration 74234-1.). In 2007, the USDA also approved an OmniLytics bacteriophage product designed to be sprayed, misted or used as washing solution on cattle, in order to reduce the concentration of *Escherichia coli.* These approvals illustrate the level of concern of the regulatory authorities in relation to the microbial threat and the need for consumers to have an alternative way of assessing microbiologically safe products.

Some reluctance in the use of live viruses can be overcome by diffusing the knowledge that phages are ubiquitous, already occurring naturally in all the places bacteria are found, namely in foods (Inal 2003). Scepticism shared by many scientists regarding the potential of phage therapy in the treatment of bacterial diseases was based on the poor quality of the science behind the early clinical trials (Sulakvelidze and Morris 2001). The sources and the methods of production of the bacteriophage suspensions, the characteristics of the phage formulations such as the volume to be administered and the concentration of the phage particles along with the frequency of administration were not documented well enough (Sulakvelidze and Morris 2001; Morrison and Rainnie 2004). Recent successes of phage therapy experiments conducted in controlled conditions provided strong scientific evidence in favor of bacteriophage therapy. Rigorous standardization of experimental designs throughout documentation and rigorous control must be assured in all studies (Morrison and Rainnie 2004).

The actual microbial threat in aquaculture and the need to fulfilling demands of consumer will ultimately conduct to the approval of more bacteriophage-based antimicrobials.

Milestones of phage therapy in aquaculture

Phage applications began in medicine, agriculture, food industry and waste water treatment. Phage therapy and prophylaxis have already been tested in a variety of species, including mice, rabbits and sheep. In aquaculture, phages are only now being more broadly explored, and the concept of phage therapy is extending to diverse aquatic organisms, in addition to fish, whether or not they originate from aquaculture.

Some of the achievements related to bacteriophage therapy in aquaculture, including phage isolation and potential applications, are summarized in Table 2. This compilation of information contributes to the identification of some of the most relevant aspects of the use of phages as antimicrobial agents in aquaculture while helping to understand the evolution of phage therapy in aquaculture.

Fish

The most successful use of phage therapy in aquaculture, already in practice, has been demonstrated in the control of fish pathogens (Nakai and Park 2002; Inal 2003; Park and Nakai 2003; Skurnik and Strauch 2006). The applicability of phages for biological control of fish pathogens was first mentioned in 1981, and the earliest attempt to verify the therapeutic potential application of phages to aquacultured japanese eel

Table 2 Research on bacteriophages potential in aquaculture

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Japanese eel *Anguilla Japonica*	*Edwardsiella tarda*	Edwardsiellosis	Phages ET-1	Pond water in Taiwan	In vitro experiment	Mortality (92.6%) affected 25 of 27 *E. tarda* strains and reduced the bacterial count to less than 0.15% when the bacterial suspension of 1.2×10^{12} cells ml^{-1} was infected with phages ET-1	Wu and Chao (1982), Yamamoto and Maegawa (2008)
Yellowtail *Seriola quinqueradiata*	*Lactococcus garvieae* formerly *Enterococcus seriolicida* (Eldar et al. 1996)	Lactococcosis	*Siphoviridae* (PLgY)	Cultures of *L. garvieae* isolated from diseased yellowtail		Of 26 strains of *L. garvieae* examined, 24 were sensitive to the phage but 2 strains of *L. garvieae* and another 22 species including fish- and shellfish-pathogenic bacteria (used to determine the host range of the phage) were not	Park et al. (1997, 1998)

Table 2 continued

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Yellowtail *Seriola quinqueradiata*	*Lactococcus garvieae* formerly *Enterococcus seriolicida* (Eldar et al. 1996)	Lactococcosis	*Siphoviridae* (PLgY-16, PLgY-30 and PLgW-1)	PLgW-1: natural seawater PLgY-16 and PLgY-30: *L. garvieae* culture isolated from diseased yellowtail	In vitro survival: phages inoculated in the test media (10^4 to 10^5 pfu ml^{-1} g^{-1}) In vivo survival and phage treatment experiment: administration of PLgY-16 intraperitoneally ($10^{7.5}$ pfu $fish^{-1}$ and $10^{7.2}$ pfu $fish^{-1}$) and orally ($10^{7.4}$ pfu g^{-1} and $10^{7.9}$ pfu g^{-1})	Anti-*L. garvieae* phages persisted well in various physicochemical and biological conditions, except for low acidity. The survival rate was much higher in yellowtail that received intraperitoneal injection of the phage after challenged with *L. garvieae* and higher protective effects were achieved by injections administered at the earlier time. Both intraperitoneally and orally administered phage had a protective effect on experimental *L. garvieae* infection	Nakai et al. (1999)

Table 2 continued

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Ayu fish *Plecoglossus altivelis*	*Pseudomonas plecoglossicida*	Bacterial hemorrhagic ascites disease	*Myoviridae* (PPpW-3) and *Podoviridae* (PPpW-4)	Diseased ayu and water from fish farms	Oral administration of phage-impregnated feed (10^7 pfu g^{-1})	Protective effect against experimentally induced infection Inhibition of bacterial growth in water (prophylactic use of phages to prevent horizontal transmission of the pathogen)	Park et al. (2000), Nakai and Park (2002)
Japanese eel *Anguilla japonica*	*Aeromonas hydrophila* and *Edwardsiella tarda*	Hemorrhagic septicaemia and edwardsiellosis	Several bacteriophages of *A. hydrophila* and *E. tarda*	Pond water samples of Southern Taiwan	Pure culture of host-phages. Pond water experiment: *A. hydrophila* (6×10^5 cfu ml^{-1}) prior to phage introduction in the water	In pure culture, the host concentration was reduced 3 orders of magnitude in 2 h when the multiplicity of infection (moi) was above 11.5 at 25°C. In the pond water the number of *A. hydrophila* dropped 250 folds at phage moi of 0.23 in 8 h with simultaneous phage multiplication to 10^6 pfu ml^{-1} in the water. The surviving hosts (85%) were still vulnerable to the phage. *E. tarda* dropped rapidly even in the absence of phage in the pond water after 48 h	Hsu et al. (2000)

Table 2 continued

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	Luminous vibriosis	*Myoviridae* (VHLM)	Extracted from a toxin-producing strain of *V. harveyi* isolated from moribund Prawn larvae		VHML showed a narrow host range and an apparent preference for *V. harveyi* rather than other 63 *Vibrio* isolates and 10 other genera	Oakey and Owens (2000), Oakey et al. (2002)
Ayu fish *Plecoglossus altivelis*	*Pseudomonas plecoglossicida* PTH-9802 strain	Bacterial hemorrhagic ascites disease	*Podoviridae* (PPpW-3), *Myoviridae* (PPpW-4) and a mixture (PPpW-3/W-4)	Diseased ayu and pond water	In vitro and in vivo experiments, the last using laboratory tanks and a commercial fish culture pond. Oral administration of phage-impregnated feed (10^7 pfu fish^{-1})	In vitro study showed that PPpW-4 inhibited the growth of *P. plecoglossicida* and a mixture of the phages induced the highest inhibitory activity. High protection against water-borne infection was also showed. Neither phage-resistant organisms nor phage-neutralizing antibodies were detected in diseased fish or apparently healthy fish	Park and Nakai (2003)
Brook trout *Oncorhynchus fontinalis* formerly, *Salvelinus fontinalis*	*Aeromonas salmonicida* HER 1107	Furunculosis	Bacteriophage HER 110		Addition in the open water of the aquarium of bacteriophage suspensions (10^9 pfu ml^{-1})	Delayed of the infection (7 days). More than one phage could infect the bacteria strain and resistant mutants to phage HER 110 were sensitive to other phages, had a slower generation time, and very low success of replating	Imbeault et al. (2006)

Table 2 continued

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	Luminous vibriosis	*Siphoviridae*	Water from shrimp farm of the West coast of India	Eighteen-day-old shrimp were challenged with the bacteria (10^5 cells ml^{-1}) Laboratory trial: (1) bacteriophage suspension (10^9 pfu ml^{-1}) added initially and after 24 h (another 0.1 ml); (2) only once initially with 0.1 ml of the phage suspension; (3) no addition Hatchery trial (in triplicates): (1) treatment with bacteriophage (10^9 pfu ml^{-1}) at the rate of 200 ppm daily so that phage concentration in the water was 2×10^5 pfu ml^{-1}; (2) treatment with antibiotics (Oxytetracycline 5 ppm, Kanamycin 10 ppm daily); (3) no treatment	Laboratory trial showed that survival of *Penaeus monodon* larvae was enhanced 80% with treatment with two doses of bacteriophage as compared with the control (25%) Hatchery trial: survival in the control tank was only 17%, while in antibiotic treated tanks was 40% and in the bacteriophage treated tank was 86% This study shows that bacteriophages have excellent potential in management of luminous vibriosis in aquaculture systems	Vinod et al. (2006)

Table 2 continued

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	Luminous vibriosis	Lytic bacteriophages against *Vibrio harvevi*, two from *Siphoviridae*	Three from oyster tissue and one from shrimp hatchery water	Tanks with post-larval 5 stage larvae, showing luminescence and mortality were used. Bacteriophage treatment (two tanks): one suspension (2×10^6 pfu ml^{-1}) was added by day following the order: Viha10, Viha8, Viha10 and Viha8 Chemotherapy (two tanks): oxytetracycline (5 mg L^{-1}) and kanamycin (10 mg L^{-1})	Bacteriophage treatment resulted in over 85% survival of *Penaeus monodon* larvae The normal hatchery practice of antibiotic treatment resulted in a survival ranged from 65 to 68% This study shows that bacteriophages could be used for biocontrol of *V. harveyi*	Karunasagar et al. (2007)
Atlantic salmon *Salmo salar*	*Aeromonas salmonicida* subsp. *salmonicida* 78027	Furunculosis	Combination of bacteriophages O, R and B		Juveniles of Atlantic salmon were challenged with the bacteria by intraperitoneal injection Phage treatments: (1) intraperitoneal injection (1.9×10^8 pfu $fish^{-1}$); (2) oral administration (1.88×10^5 pfu g^{-1} $fish^{-1}$ daily for 30 days), bath (1.04×10^5 ml^{-1} daily for 30 days) and intraperitoneal injection (6.25×10^7 pfu $fish^{-1}$) Chemotherapy: oxolinic acid (10 mg kg^{-1} bw^{-1} day^{-1}) for 10 days	Fish injected with bacteriophage, immediately after challenge with the bacteria, died at a significantly slower rate than those that were either not treated with bacteriophage, or treated 24 h post-challenge but 100% mortality was observed. The effects of oral, bath and intraperitoneal injection phage treatment were compared with chemotherapy using an indirect cohabitation challenge. No protection was offered by any of the bacteriophage treatments, compared to the positive challenge group, although significant protection was offered by the oxolinic acid treatment. It was also shown that bacteriophage-resistant bacteria isolates could be recovered from dead juveniles in all the treatment groups	Verner-Jeffreys et al. (2007)

Table 2 continued

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Japanese flounder *Paralichthys olivaceus*	*Streptococcus iniae*	Streptococcosis		Fish culture environments	Fish were injected intraperitoneally with *S. iniae* PSi402 and 1 h later intraperitoneally injected with a mixture of two or four phage isolates	Mortality of fish receiving phages was significantly lower than those of control fish without phage treatment in all trials. However, as phage-resistant *S. iniae* were frequently isolated from dead fish in the phage-treated group, further investigations are required to establish phage therapy of the disease	Matsuoka et al. (2007)
Penaeid shrimp	*Vibrio harveyi*	Luminous vibriosis	Seven bacteriophages specific to *Vibrio harveyi* (Viha1 to Viha7), six from *Siphoviridae* and one *Myoviridae* (Viha4)	Coastal aquaculture systems like shrimp farms, hatcheries and tidal creeks along the East and West coast of India		All the phages were found to be highly lytic for *V. harveyi* and had different lytic spectrum for the large number of isolates tested Three of the phages (Viha1, Viha3 and Viha7) caused 65% of the strains to lyse while Viha2, Viha4 and Viha6 caused 40% of the host strains to lyse. Only Viha5 had a narrow spectrum (14%) Six of the seven phages isolated had a broad lytic spectrum and could be potential candidates for biocontrol of *V. harveyi* in aquaculture systems	Shivu et al. (2007)

Table 2 continued

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Shrimp	*Vibrio harveyi*	Luminous vibriosis	*Siphoviridae* (VH1 to VH8)	Shrimp farm	In vitro experiment	All the isolates of bacteriophage (VH1-VH8) caused lysis of the host bacterial cells within 2 h. The propagation curve for each phage shows a burst time started from 1-10 h. Bacteriophages of *Vibrio* sp. might be effectively used in vivo as biological agents to control these pathogenic bacteria in aquaculture systems	Srinivasan et al. (2007)
Catfish	*Edwardsiella ictaluri*	Enteric septicemia	*Siphoviridae* (ΦeiDWF and ΦeiAU)			Initial characterization of these bacteriophages has demonstrated their potential use as biotherapeutic and diagnostic agents associated with enteric septicemia of catfish	Walakira et al. (2008)
Rainbow trout *Oncorhynchus mykiss* and other species of trouts	28 different strains of *Flavobacterium psychrophilum*	Rainbow trout fry syndrome (RTFS) and bacterial coldwater disease (BCWD)	Twenty-two bacteriophages (FpV-1 to FpV-22)	Water and sediment samples from Danish freshwater rainbow trout farms		The most potent phages belonged to genome size group 1 (FpV-1 to FpV-4) and group 2 (FpV-5 to FpV-11), which together infected 24 of the 28 *F. psychrophilum* strains examined, including strain 950106-1/1, which is highly pathogenic to rainbow trout. This range of host strains was mainly covered by phages FpV-5, FpV-6, pV-8, FpV-9, and FpV-11. Moreover, FpV-9 also had the highest infection efficiency of the analyzed phages, and apparently, a combination of FpV-4, FpV-9, and FpV-21 would seem to constitute the most potent cocktail of the isolated phages, together infecting 24 of the 27 Danish *F. psychrophilum* strains, with 20 of the 24 phage-host interactions being lytic	Stenholm et al. (2008)
Ayu fish *Plecoglossus altivelis altivelis*	*Flavobacterium psychrophilum*	Systemic bacterial coldwater disease (BCWD)	*Myoviridae* (PFpW-3, PFpC-Y), *Podoviridae* (PFpW-6, PFpW-7), and *Siphoviridae* (PFpW-8)	Pond water from Japanese ayu farms		PFpW-3 had high infectivity for *F. psychrophilum* isolated from ayu and other fish and proved to be efficient for the reduction of bacterial growth	Kim et al. (2010)

Table 2 continued

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Shrimp	*Vibrio harveyi* CS101	Luminous vibriosis	*Siphoviridae* (Phage PW2)	Shrimp pond water		The phage adsorption rate increased rapidly in the first 15 min of infection to 80% and continued to increase to 90% within 30 min of infection. The stability of phage PW2 was dependent on temperature and pH. It was inactivated by heating at 90°C for 30 min and by treating at pH 2, 3, 11and 12. From its one-step growth curve, latent and burst periods were 30 and 120 min, respectively with a burst size of about 78 pfu per infected center. Six structural proteins were detected	Phumkhachorn and Rattanachaikunsopon (2010)
Phyllosoma larvae of the tropical rock lobster Panulirus ornatus	*Vibrio harveyi*	Luminous vibriosis	Six bacteriophages from Siphoviridae (VhCCS-01, VhCCS-02, VhCCS-04, VhCCS-06, VhCCS-17 and VhCCS-20) and two from *Myoviridae* (VhCCS-19 and VhCCS-21)	Water samples from discharge channels and grow-out ponds of a prawn farm	Nine cultures of *V. harveyi* strain 12 Bacteriophage treatments (in triplicate): (1) addition of phage VhCCS-06 (1 ml) 2 h after inoculation; (2) addition of phage VhCCS-06 (1 ml) 6 h after inoculation	The *Myoviridae* (VhCCS-19 and VhCCS-21) were lysogenic and appeared to induce bacteriocin production in a limited number of host bacteria (*V. harveyi* strain 12) One *Siphoviridae* phage (VhCCS-06) could delay the entry of a broth culture of *V. harveyi* strain 12 into exponential growth, but could not prevent the overall growth of the bacterial strain. This effect was most likely because of multiplication of phage-resistant cells	Crothers-Stomps et al. (2010)

Table 2 continued

Aquaculture product	Etiologic agent	Infection disease	Bacteriophage	Bacteriophage source	Bacteriophage administration	Result	Reference
Catfish *Clarias batrachus*	*Flavobacterium columnare*	Columnaris disease	Nine bacteriophages (FCP1–FCP9), FCP1 belong to *Podovariedae*	Water and bottom sediments	FCP1 phage was inoculated intramuscularly with virulent bacterial isolate (FC8) and post inoculated with FCP1phage at 10^8:10^6 :: cfu: pfu through intramuscular, immersion and oral administration	Protective effect (100% survival) After 6 h of phage treatment host bacterium concentration reduced (less than 10^{-3} cfu ml^{-1}) in the sera, gill, liver and kidney. The sera of dose 1 (4.55×10^6 pfu ml^{-1}) and dose 2 (9.15×10^6 pfu ml^{-1}) treated fishes mean $\log_{10}$ cfu value reduced by 3 logs (58.39%) and 5 logs (73.77%) at 96 h, respectively	Prasad et al. (2011)
Catfish	*Edwardsiella ictaluri*	Enteric septicemia	*Siphoviridae* (ΦeiDWF, ΦeiAU and ΦeiMSLS)	Water of aquaculture ponds		The genomic analysis revealed that these are virulent phages, lacking the capacity for lysogeny or expression of virulence genes	Carrias et al. (2011)

(*Anguilla japonica*) was reported 1 year later (Wu et al. 1981; Wu and Chao 1982). The results showed that *Edwardsiella tarda* could be inactivated in the water system, allowing for an economical method of disease control (Wu and Chao 1982). Although some other attempts were made, reliable field experiments on phage therapy in fish were developed in 1997 when the protective effect of bacteriophage on experimental *L. garvieae* infections in yellowtail (*Seriola quinqueradiata)* was observed (Nakai et al. 1999). At that time, there was a commercial vaccine available against lactococcosis, but yellowtail aquaculture had no alternatives to chemotherapy and drug-resistant strains of *L. garvieae* were frequent (Nakai et al. 1999). Primarily, the virulent bacteriophage specific to *L. garvieae*, designated as PLgY, was isolated from diseased fish and identified as a member of the family *Siphoviridae* (Park et al. 1997). The *L. garvieae* strains were then distributed into groups according to the susceptibility to a set of phages isolated from fish and culture environments (Park et al. 1998).

The application of bacteriophages in cultured ayu fish (*Plecoglossus altivelis*) was of particular importance, it enabled the control of *P. plecoglossicida* when licensed chemotherapeutic compounds were still not licensed for aquaculture and the control was only addressed by reducing the predisposing factors could be used. The most relevant findings that lead to the proposal of the use of phages for prophylactic and therapeutic purposes in fish pathology were that: (1) phage-impregnated food protected fish against experimental infection, indicating high level of phage activity in vivo (Park et al. 2000; Park and Nakai 2003); (2) phage suspension could be also used in water to prevent transmission of pathogens; (3) neither phage-resistant organisms nor phage-neutralizing antibodies were detected in diseased fish or apparently healthy fish and (4) phages reduced the frequency of infections outbreaks and fish mortality (Park and Nakai 2003).

The results obtained with these fish models had served as reference for biocontrol approaches of a large number of species of fish in aquaculture thus indicating the potential of phage to control bacterial diseases of fish from aquaculture (Nakai and Park 2002). Imbeault and colleagues suggested that bacteriophage combinations could be successfully used in preventive programs on fish farms (Imbeault et al. 2006). Although prophylactic use of phages was recommended, bacteriophage therapy failed to treat furunculosis caused by *Aeromonas salmonicida* in farmed brook trout (Imbeault et al. 2006) and in Atlantic salmon (Verner-Jeffreys et al. 2007). Although safety problems associated with both approaches were not found, these unsuccessful trials suggested that furunculosis is not readily controllable by application of bacteriophage (Imbeault et al. 2006; Verner-Jeffreys et al. 2007). However, published reports on the etiologic agent of furunculosis presented the possibility of phage treatment (Ackermann et al. 1985; Hidaka and Kawaguchi 1986; Kay and Trust 1991; Olivier 1992; Wiklund and Dalsgaard 1998; Roberts et al. 2002). Even though these studies have provided considerable documentation of the existence and characteristics of bacteriophages of *A. salmonicida* subspecies *salmonicida*, it is unclear whether the host range of the *A. salmonicida* phages extends to any of the various atypical *A. salmonicida* subspecies (Morrison and Rainnie 2004), and the treatment of furunculosis is still a challenge to phage therapy. Nevertheless, bacteriophage therapy was successfully used to treat columnaris disease in the catfish, *Clarias batrachusm*, caused by *Flavobacterium columnare*. Results demonstrated that the phage FCP1 exhibited broader host range to lyse 9 out of 15 isolates of *F. columnare*. After treatment gross symptoms disappear, bacteriological as well as phage detection tests were negative, and all the experimentally infected *C. batrachus* survived (Prasad et al. 2011).

Crustaceans

Shrimps

Antibiotics are commonly used in shrimp farming to prevent or treat disease outbreaks (Holmström et al. 2003). *V. harveyi* has become recognized as a serious cause of disease, particularly of marine invertebrates, namely the economically important penaeid shrimp (Karunasagar et al. 2004; Austin and Zhang 2006). Antibiotics used in the hatchery are sometimes ineffective in controlling luminous bacteria, when antibiotic-resistant *V. harveyi* emerges in larval tanks causing serious mortality in *P. monodon* larvae (Lavilla-Pitogo et al. 1990; Karunasagar et al. 1994). It is estimated that many of the shrimp farmers use different antibiotics prophylactically, some on a daily basis (Holmström et al. 2003). There is evidence of antibiotic resistance in shrimp aquaculture (Tendencia and de la Peña 2001), but data on the use of phage therapy applied to invertebrates, like shrimp, are very scarce. A bacteriophage isolated from hatchery water proved to be infect *V. harveyi*, suggesting its potential as a biocontrol agent of luminous vibriosis. In laboratory microcosms, the addition of bacteriophages decreased the concentration of *V. harveyi* by about 2–3 log units (Karunasagar et al. 2005). Similar results were obtained in an invertebrate hatchery infected with luminous vibriosis (Vinod et al. 2006). Both reports showed that bacteriophages could be used for biocontrol of *V. harveyi* and that bacteriophage therapy was an effective alternative to antibiotics in the control of luminous vibriosis in shrimp hatchery systems (Karunasagar et al. 2007). In vitro experiment confirmed that bacteriophages could be effectively used in vivo as biological agents to control *Vibrio* sp. in aquaculture systems (Srinivasan et al. 2007). However, studies on the distribution of luminescent *V. harveyi* and their bacteriophages in shrimp hatchery indicated that the occurrence of luminescent bacteria, even in low concentrations during early larval stages, would lead to the development of luminous vibriosis, despite the presence of bacteriophages in the larval rearing tanks (Chrisolite et al. 2008). The first report on the lytic spectrum of naturally occurring phages against a large collection of host bacteria obtained from different locations around the world identified six bacteriophages (Viha1, Viha2, Viha3, Viha4, Viha6, Viha7) with a wide spectrum of activity against *V. harveyi,* suggesting their potential as agents for biocontrol of luminous vibriosis in aquaculture environments (Shivu et al. 2007). Phage PW2, a new lytic phage infecting *V. harveyi* CS101, was isolated and characterized in order to investigate its lytic property toward its host bacterium under controlled conditions in the laboratory (Phumkhachorn and Rattanachaikunsopon 2010). Some successful experiments using bacteriophages for the biocontrol of luminous vibriosis, as well as studies that characterized bacteriophages that could be used in the control of this disease, have been reported in the literature (Table 2). Future work will imply obtaining consistent results to corroborate the application of bacteriophage as an effective way to control luminous vibriosis in shrimps and extending research to other organisms to which this disease is associated with. The versatility of phage therapy to control microbial infections that occur in different organisms (vertebrates or invertebrates), at various stages (from eggs to broodstock) as well as in laboratory, tanks or field applications, was showed in experiments made with shrimp larvae showing a promising potential for phage therapy (Vinod et al. 2006).

Lobsters

Bacteriophage therapy was also proposed, among other alternatives, as a technique to control and remove the pathogenic *Vibrio* spp. from the larval cultures of the tropical rock

lobster, *Panulirus ornatus* (Payne 2007). *V. harveyi* has been found to be associated with diseases in spiny lobster (Vinod et al. 2006). Crothers-Stomps and colleagues demonstrated that from eight bacteriophages (six phage belonged to the family Siphoviridae and two belong to the family Myoviridae), only one bacteriophage from the family Siphoviridae had a clear lytic activity against *V. harveyi* and no apparent transducing properties. Also, they have identified phage resistance as a major constraint to the use of phage therapy in aquaculture since bacteria were not completely eliminated (Crothers-Stomps et al. 2010).

Bivalve molluscs

It has been suggested that phage treatment could be a useful approach to control *Vibrio splendidus* infection (Sugumar et al. 1998) in cultured larvae of the Pacific oyster, *Crassostrea gigas* (Park and Nakai 2003) and more generally for treatment of bacterial infections in molluscan aquaculture production (Berthe 2005). *V. harveyi* has been associated with diseases in pearl oysters (Pass et al. 1987) but phages as an antimicrobial strategy to overcome this disease was not yet demonstrated. Reports on microbial control with phages for either these or any other bivalve species are still not available in scientific literature.

Characterization of bacteriophages for phage therapy

Several bacteriophages related to major fish diseases had been identified and characterized (Rodgers et al. 1981; Stevenson and Airdrie 1984; Merino et al. 1990; Yuksel et al. 2001; Munro et al. 2003) but not envisaging their application in phage therapy against important aquatic animal pathogens.

Male-specific RNA (F-RNA) bacteriophages (Doré et al. 2000; Doré et al. 2003), somatic coliphages (Chai et al. 1994; Albert et al. 1995; Legnani et al. 1998; Nanni et al. 2000; Miossec et al. 2001) and bacteriophages infecting *Bacteroides fragilis*, have already been associated with bivalves (Hernroth et al. 2002). These bacteriophages have been proposed as putative indicators of viral contamination in shellfish since more representative and accurate indicators to improve the microbial control of shellfish are essential (Beril et al. 1996; Chung et al. 1998; Grabow 2001). However, their potential as therapeutic agents to control bacteria that cause infections in bivalves or that contaminates this food product is not yet explored.

Some phages with strong lytic potential against different pathogenic bacteria, which are considered to be responsible for serious economic damage in aquaculture, were isolated and characterized (Table 1). The fish pathogen *Piscirickettsia salmonis* showed the presence of bacteria containing phage particles attached to the cell wall that appeared to eventually lyse the cell (Yuksel et al. 2001). The characterization of those bacteriophages associated with *P. salmonis* was being done in the perspective of using those bacteriophages for the biological control of diseases of cultured fish (Yuksel et al. 2001). Information on bacteriophages isolation and characterization could be valuable to the evaluation of the therapeutic success of the use of these phages individually or as a cocktail of phages, in the control of bacterial infections in aquaculture facilities (Shivu et al. 2007; Stenholm et al. 2008; Walakira et al. 2008; Kim et al. 2010; Phumkhachorn and Rattanachaikunsopon 2010; Carrias et al. 2011).

Lysins: extensions of the "phage therapy" concept

Now that the mechanisms by which phages lyse bacteria are becoming elucidated, the partial use of specific phage components involved in bacterial lysis as an alternative to the whole bacteriophage particles, is being explored (Inal 2003; O'Flaherty et al. 2009). It would be easier to gain public acceptance of phage therapy by using one viral lytic enzyme rather than using a whole virus. Phage-specific lysins or phage peptides blocking cell-wall synthesis are being isolated and tested as an alternative to using whole bacteriophage particles for potential therapy (Inal 2003). Most tailed phages encode peptidoglycan hydrolase (endolysin or lysin) involved in the release of the progeny at the final stage of lytic cycle. Lysin is able to degrade peptidoglycan directly, exerting a bacteriolytic effect within several seconds of administration. It can also destroy the cell walls of nongrowing bacteria, which are insensitive to many antibiotics (Matsuzaki et al. 2005; Hermoso et al. 2007). The simultaneous administration of two lysins that have different peptidoglycan cutting sites has a synergistic effect. With the exception of the lysin of an enterococcal phage, lysins are fairly specific for bacterial species as well as phages themselves, indicating that phage lysin can very likely eliminate the targeted bacteria without disturbing the normal flora (Matsuzaki et al. 2005). Other advantages and drawbacks of the use of these lytic enzymes were summarized elsewhere (O'Flaherty et al. 2009). Various lysins have been proven efficient in inactivating *Bacillus anthracis* (Schuch et al. 2002; Yoong et al. 2006), *Streptococcus pyogenes* (a group A streptococcus) (Nelson et al. 2001), *Enterococcus faecalis* and *Enterococcus faecium* (Yoong et al. 2004), *Staphylococcus aureus* (Rashel et al. 2007) and *Streptococcus pneumoniae* (Loeffler et al. 2001; Jado et al. 2003; Loeffler et al. 2003; Entenza et al. 2005; McCullers et al. 2007; Grandgirard et al. 2008) both in vitro and in animal models naturally or artificially infected with these pathogens but research in the field of aquaculture have not yet been done.

Future perspectives: an example

Freshwater finfish represents half of global aquaculture production (54%) being molluscs the second more produced aquaculture item in the world (24%) (FAO 2009). Crustaceans come next in production relevance, represented mostly by penaeid shrimps and grapsid crabs (FAO 2006, 2009).

In mollusc and shrimp aquaculture, infectious disease is the most devastating problem (Mialhe et al. 1995). Molluscan aquaculture production represents a particular situation, since very few ways to reduce disease in commercially exploited molluscs have been proposed (Berthe 2005). The production of molluscs occurs in the natural environment using the primary productivity of the surrounding waters as food source, not requiring any food inputs for growth or implying any additional costs (Helm and Bourne 2004; Berthe 2005; FAO 2006). This condition strongly limits the chemotherapeutic possibilities in an open system since it is neither practical nor safe for the surrounding environment, because of the quantity needed. Contradictory results on the use antibiotics on bivalves have been reported (Le Pennec and Prieur 1977; Berthe 2005; Giraud et al. 2006). Furthermore, some antibiotics could affect larval development limiting both larvae and bivalve production (Nicolas et al. 1996). Other alternatives to chemotherapy in bivalves have been proposed (Verschuere et al. 2000; Prado et al. 2010). Research into the use of phage therapy with regard to shrimp is still in its infancy (Karunasagar et al. 1994; Vinod et al. 2006; Karunasagar et al. 2007), and the use of bacteriophages on molluscs is still not a common

practice. Since bivalve molluscs are passive filter-feeders, along with the oxygen and nutrients, they accumulate various chemical and biological contaminants (Oliveira et al. 2011). Autochthonous and allochthonous bacteria are a source of nutrients and a challenge to the innate immune system of bivalve molluscs (Girón-Pérez 2010). Like bivalve molluscs, corals have no adaptative immune system and grow in open waters (coral reef). A pioneering research about the application of lytic bacteriophages of *Vibrio coralliilyticus* and *Thalosomonas loyaeana* as an alternative for preventing or treating infectious diseases of *Pocillopora damicornis* and *Favia favus* corals, respectively, demonstrated that diseases in both corals could be controlled by the respective pathogen-specific phage (Efrony et al. 2007). This research was of particular relevance since it suggested phage application in an open system such as a coral reef attending that (1) the appropriate time to add the phage should be studied, (2) the development of phage resistance should be investigated and (3) the number of phages needed for a field trial should be quantified and produced. It was estimated that if 10^3 bacteriophage per ml would prevent the spread of the disease, a commercial fermentor of 100 m^3 would provide phages suspensions to treat large reef areas of 1,000 km^2 (Efrony et al. 2007). This research highlighted the potential of the use of bacteriophages in molluscan aquaculture in the view of the resemblance of these organisms in some conjectural facts.

As far as it has been studied, bacteriophages found in the environment are able to eliminate natural occurring planktonic bacteria but also pathogenic bacteria (Nakai and Park 2002). So, the use of bacteriophages in mollusc might: (1) decrease bacterial infection and therefore economic losses and (2) decrease bacterial contamination improving the safety of mollusc products for human consumption.

Phage numbers can decline in an open system even if phage application is done with high ratios of phages to bacteria as it already has been suggested (Efrony et al. 2007). Pre-harvest reduction of bacteria in bivalves from natural growing areas or produced in an open system by the use of phages might not be effective due to the natural decay of phages in the environment. Exposure to sunlight, UV radiation, desiccation, and various chemical and biological antagonists might contribute to this decline (Wommack et al. 1996; Wilhelm et al. 1998; Sinton et al. 1999). Phage viable counts typically decline in many ecosystems, given an absence of specific host bacteria.

A variety of infectious diseases occur locally in a diversity of fish and shellfish (Nakai and Park 2002). Therefore, for prophylactic measures to be addressed locally: (1) a rapid and well-designed diagnostic scheme for detection and identification of the existing pathogens causing diseases must be developed; (2) a detailed characterization of isolated phages must be made (their adsorption rate, lytic potential, interaction with host, among others) and (3) a well-achieved knowledge of the surrounding environment, as well as the seasonal dynamics of bacterial communities, must be obtained (Mialhe et al. 1995; Stenholm et al. 2008; Pereira et al. 2011).

The bioaccumulation of harmful microorganisms and the fact that it is traditionally consumed whole, raw or lightly cooked make shellfish a particular case for foodborne disease and a high-risk food product (Lees 2000). Regulations for monitoring microbial quality of harvesting areas of mollusc bivalves aim to safeguard public health. Restriction to bivalve harvesting is often applied when the area does not meet the required sanitary standards (Oliveira et al. 2011). The quality of harvesting areas could be indirectly improved by the use of bacteriophages in wastewater treatment plants, reducing the anthropogenic bacterial inputs in these areas. Indeed, the use of phages for wastewater treatment processes was already suggested (Withey et al. 2005).

Depuration and transposition are the only post-harvested "natural" ways of reducing the microbial loads of bivalve molluscs for human consumption (Oliveira et al. 2011). Since depuration occurs in the confinement of commercial depuration tanks, phages could be added as an additional factor of improvement of the sanitary quality of bivalves and for acceleration of the depuration process, achieving safer products for human consumption. The advantage of using phage therapy in food products, which will be consumed raw or lightly cooked and for which the appearance is overvalued, relies on the fact that it will look natural as if no treatment was given: no flavor, aroma, preservatives or nutritional value will be added.

Conclusions

Bacteriophage therapy has been the subject of intensive research in many fields, but phage therapy against bacterial diseases in aquaculture is not yet fully investigated. Aquaculture has a great impact on the economy and involves mainly the production of different species of fish, crustaceans and molluscs. Most of the economic losses in aquaculture are associated with disease outbreaks caused by pathogenic bacteria. Bacteriophage therapy in fish pathogens from aquaculture has been already implemented. However, little information exists on phage therapy on other aquatic organisms like molluscs and crustaceans. In this work, the particular case of bivalve molluscs is discussed. Indeed, research is required in all taxonomic groups of aquaculture, particularly those to which less attention has been given, in order to minimize the impacts of disease, stimulating production and protecting public health.

Bacteriophage therapy has been suggested as an alternative for the prevention and treatment of microbial diseases in aquaculture. The use of naturally occurring bacteriophages as antimicrobial agents in aquatic environments for fish diseases and other infections showed both successful and unsuccessful results. Although phage therapy is showing to be promising, caution must be taken with the highlighted issues in this work. The impact of this biocontrol technique in the aquaculture system and on the respective product must be taken into account. Even though it is less likely that bacteriophages pose a selective pressure for the development of resistance than conventional antibacterial compounds do, bacteria resistance should not be neglected. Both the cost of bacteriophage treatment and the delivery route should be considered. Additionally, the success of the administration of phage cocktails combined with antibiotic therapy as well as the use of lytic enzymes in aquaculture must be confirmed. Advantages and drawbacks are inherent to any antimicrobial approach, and a good management strategy would consider the use of several techniques in rotation. This could prevent and minimize the most expensive drawbacks of the actual control strategy in aquaculture. Approval by the authorities is required for the expansion of phage therapy in aquaculture. The fact that phage sprays are already approved for use in other food items shows that phage therapy is becoming a reality and that concerns related with the impacts of chemotherapy can be overcome.

The application of phages in aquaculture has some advantages mainly related to the direct attack of the infection, the versatility of administration routes, the variety of aquaculture organisms to which it can be applied (vertebrates or invertebrates, at various stages of development) as well as the possibility of use in closed or open systems. The potential for naturally occurring lytic bacteriophages to be used in therapy and prophylaxis of bacterial diseases in aquaculture is therefore wide and promising.

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